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(54) **CHEMICAL APPROACHES FOR GENERATION OF INDUCED PLURIPOTENT STEM CELLS**

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C12N 15/85	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 5/0696*** (2013.01); ***C12N 15/85*** (2013.01); *C12N 2500/14* (2013.01); *C12N 2501/01* (2013.01); *C12N 2501/065* (2013.01); *C12N 2501/15* (2013.01); *C12N 2501/602* (2013.01); *C12N 2501/603* (2013.01); *C12N 2501/604* (2013.01); *C12N 2501/605* (2013.01); *C12N 2501/608* (2013.01); *C12N 2501/727* (2013.01); *C12N 2506/08* (2013.01); *C12N 2506/094* (2013.01); *C12N 2506/1307* (2013.01); *C12N 2506/28* (2013.01); *C12N 2510/00* (2013.01)

(58) **Field of Classification Search**

CPC C12N 5/0696; C12N 15/86

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,824,837	A	10/1998	Chen et al.
5,843,780	A	12/1998	Thomson
6,200,806	B1	3/2001	Thomson
7,029,913	B2	4/2006	Thomson
7,265,138	B2	9/2007	Doherty et al.
8,298,825	B1	10/2012	Hochedlinger et al.
8,603,818	B1	12/2013	Hochedlinger et al.
8,906,677	B2	12/2014	Li et al.
9,005,968	B2	4/2015	Lin et al.
9,068,170	B2	6/2015	Zhou et al.
9,394,524	B2	7/2016	Lin et al.
2002/0142457	A1	10/2002	Umezawa et al.
2004/0157324	A1	8/2004	Spradling et al.
2006/0182724	A1	8/2006	Riordan
2007/0032447	A1	2/2007	Eilertsen
2007/0128719	A1	6/2007	Tseng et al.
2007/0134215	A1	6/2007	Fukuda et al.
2007/0141703	A1	6/2007	Stanley et al.
2007/0161107	A1	7/2007	Mummery et al.
2007/0172946	A1	7/2007	Smith et al.
2007/0196919	A1	8/2007	Reh et al.
2007/0254359	A1	11/2007	Rezania et al.
2007/0259423	A1	11/2007	Odorico et al.
2007/0264709	A1	11/2007	Smith et al.
2007/0269412	A1	11/2007	Kopyov
2007/0281355	A1	12/2007	Dalton et al.
2008/0066197	A1	3/2008	Ying et al.
2008/0242594	A1	10/2008	McKay et al.
2008/0268533	A1	10/2008	Dalton et al.
2009/0068742	A1	3/2009	Yamanaka
2009/0117439	A1	5/2009	Fujinami et al.
2009/0203690	A1	8/2009	Akritopoulou-Zanze et al.
2009/0227032	A1	9/2009	Yamanaka et al.
2011/0033931	A1	2/2011	Schwartz et al.
2011/0039332	A1	2/2011	Sakurada et al.
2011/0110899	A1	5/2011	Shi et al.
2012/0122212	A1	5/2012	Grskovic et al.

(Continued)

FOREIGN PATENT DOCUMENTS

CN	101356270	1/2009
EP	1970446 A1	9/2008

(Continued)

OTHER PUBLICATIONS

Li et al. Cell Stem Cell 4:16-19, Jan. 9, 2009.*

Takahashi, Kazutoshi et al.; "Induction of Pluripotent Stem Cells from Adult Human Fobroblasts by Defined Factors"; Cell, 131: 1-12 (2007).

Zhou, Hongyan et al.; Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins; Cell Stem Cell, 4: 381-384 (2009).

International Search Report and Written Opinion from PCT/US2009/037429, dated Aug. 12, 2009 (14 pages).

Beaujean et al., Dev. Biol., 2000, vol. 2000, pp. 337-354.

Debs et al., J. Biol. Chem., 1990, vol. 265, pp. 10189-10192.

(Continued)

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(74) *Attorney, Agent, or Firm* — Kilpatrick Townsend & Stockton LLP

(57) **ABSTRACT**

The present invention provides for identification and use of small molecules to induce pluripotency in mammalian cells as well as other methods of inducing pluripotency.

12 Claims, 14 Drawing Sheets

(56) **References Cited**

U.S. PATENT DOCUMENTS

2012/0129172	A1	5/2012	Okano et al.
2012/0196360	A1	8/2012	Okita et al.
2013/0273536	A1	10/2013	Shi et al.
2013/0323833	A1	12/2013	Zhu et al.
2015/0079675	A1	3/2015	Li et al.
2015/0240214	A1	8/2015	Lin et al.

FOREIGN PATENT DOCUMENTS

GB	2 436 737		10/2007
GB	2 450 603	A	12/2008
JP	2007/508026		4/2007
JP	2008/307007		12/2008
JP	2010/529851		9/2010
WO	03/095628	A2	11/2003
WO	2007/016566	A2	2/2007
WO	2007/069666		6/2007
WO	2007/113505		10/2007
WO	2008/015418	A2	2/2008
WO	2008/056173	A2	5/2008
WO	2008/088882	A2	7/2008
WO	2008/089351		7/2008
WO	2008/105630	A1	9/2008
WO	2009/006422	A1	1/2009
WO	2009/032194	A1	3/2009
WO	2009/032456	A1	3/2009
WO	2009/057831		5/2009
WO	2009/067756	A1	6/2009
WO	2009/067757	A1	6/2009
WO	2009/073523	A2	6/2009
WO	2009/117439	A1	9/2009
WO	2011/047300	A1	4/2011
WO	2011/109695	A1	9/2011

OTHER PUBLICATIONS

Ho et al., Cancer Res., 2001, vol. 61, pp. 474-477.
Krippl et al., Proc. Natl. Acad. Sci. USA, 1984, vol. 81, pp. 6988-6992.
Mi et al., Mol. Ther., 2001, vol. 4, pp. 339-347.
Pan et al., J. Biol. Chem., 2004, vol. 279, pp. 37013-37020.
Sells et al., BioTechniques, 1995, vol. 19, pp. 72-78.
Shields et al., J. Biol. Chem., 1997, vol. 272, pp. 18504-18507.
Stacey et al., Mol. Cell. Biol., 1987, vol. 7, pp. 523-527.
Wadia et al., Curr. Opin. Biotechnol., 2002, vol. 13, pp. 52-56.
Zhao et al., Cell Death and Differentiation, 2007, vol. 14, pp. 489-499.
Zheng et al., Cancer Res., 2003, vol. 63, pp. 6909-6913.
Collas et al., Reproductive BioMedicine Online: 762-770, 2006.
Djuric et al., Stem Cell Research and Therapy, 2010, 1:3.
Hudecz et al., Medicinal Research Reviews, 25(6): 679-736, 2005.
Huangfu et al., 2008, Nature Biotechnology, 26, pp. 795-797.
Huangfu et al., 2008, Nature Biotechnology, 26:1269-75.
Kubicek, et al., "Reversal of H3K9me2 by a Small-Molecule Inhibitor for the G9a Histone Methyltransferase," Molecular Cell, Feb. 2007, vol. 25, No. 3, pp. 473-481.
Meissner, et al., "Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells," Nature Biotechnology, Oct. 2007, vol. 25, No. 10, pp. 1177-1181.
Oliveri et al., Regenerative Medicine, 2(5): 795-816, Sep. 2007.
Plath et al., Nature Reviews, 12: 253-265, 2011.
Plews, et al., "Activation of Pluripotency Genes in Human Fibroblast Cells by a Novel mRNA Based Approach," PLoS ONE, Dec. 2010, vol. 5, No. 12, pp. 1-10.
Roberts et al., (PD98059 Enhanced Insulin, Cytokine, and Growth Factor Activation of Xanthine Oxidoreductase in Epithelial Cells Involves STAT3 and the Glucocoticoid Receptor, Journal of Cellular Biochemistry 2007, 101: 1567-1587.
Shi, et al., "A Combined Chemical and Genetic Approach for the Generation of Induced Pluripotent Stem Cells," Cell Stem Cell, Jun. 2008, vol. 2, No. 6, pp. 525-528.
Silva et al., PLoS Biology, 6(10): 2237-2247, Oct. 2008.
Sullivan et al., Reproductive BioMed. Online, 16(1): 41-50, Nov. 2008.
Wernig, et al., "In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state," Nature, Jul. 2007, vol. 448, No. 7151, pp. 318-324.
Aasen et al., Nat Biotechnol 26:1276-1284 (2008).
Brons et al., Nature, 2007, vol. 448, pp. 191-195.
Chambers et al., Nature, 2007, vol. 450, pp. 1230-1234.
Chen et al., Proc Natl Acad Sci USA, 2007, vol. 104, pp. 10482-10487.
Chou et al., Cell, 2008, vol. 135, pp. 449-461.
D'Amour et al., Nat Biotechnol, 2005, vol. 23, pp. 1534-1541.
Demers et al., Cloning Stem Cells, 2007, vol. 9, pp. 512-522.
Dimos et al., Science, 2008, vol. 321, pp. 1218-1221.
Dvorak et al., Stem Cells, 2005, vol. 23, pp. 1200-1211.
Feng et al, "Molecules that Promote or Enhance Reprogramming of Somatic Cells to Induced Pluripotent Stem Cells," Cell Stem Cell, 2009, 4, 301-12.
Guo et al., Development, 2009, vol. 136, pp. 1063-1069.
Han et al., Curr Stem Cell Res Ther, 2008, vol. 3, pp. 66-74.
Hayashi et al., Cell Stem Cell, 2008, vol. 3, pp. 391-401.
Hindie et al., "Structure and allosteric effects of low-molecular-weight activators on the protein kinase PDK1," Nat Chem Biol, Oct. 2009, vol. 5, No. 10, pp. 758-764.
Kanatsu-Shinohara et al., Cell, 2004, vol. 119, pp. 1001-1012.
Li et al., "Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors," Cell Stem Cell, 2009, vol. 4, pp. 16-19.
Li et al., Differentiation, 2007, vol. 75, pp. 299-307.
Li et al., "Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming," Trends Pharmacol Sci, Jan. 2010, vol. 31, No. 1, pp. 36-45.
Li et al., Stem Cells 27:2992-3000 (2009).
Lin et al., Nat Methods 6:805-808 (2009).
Lowry et al., Proc Natl Acad Sci USA, 2008, vol. 105, pp. 2883-2888.
Maherali et al., "Directly Reprogrammed Fibroblasts Show Global Epigenetic Remodeling and Widespread Tissue Contribution," Cell Stem Cell, 2007, 1, pp. 55-70.
Maherali et al., "Tgfβ Signal Inhibition Cooperates in the Induction of iPSCs and Replaces Sox2 and cMyc," Current Biology, 2009, vol. 19, pp. 1718-1723.
Muller et al., "Upping the Ante: Recent Advances in Direct Reprogramming," Mol. Ther., 2009, vol. 17, pp. 947-953.
Nakagawa et al., Nat Biotechnol, 2008, vol. 26, pp. 101-106.
Peeran I et al., EMBO J., 2007, vol. 26, pp. 4744-4755.
Ruhnke et al., Stem Cells, 2003, vol. 21, pp. 428-436.
Saha et al., Biophys. J., 2008, vol. 94, pp. 4123-4133.
Sato et al., Dev. Biol., 2003, vol. 260, pp. 404-413.
Schugar et al., Gene Ther, 2008, vol. 15, pp. 126-135.
Schulze et al., Methods Mol Biol, 2006, vol. 329, pp. 45-58.
Singh et al., Stem Cells, 2007, vol. 25, pp. 2534-2542.
Sylvester et al. (Arch Surg. 136:93-99, 2004).
Taranger et al., "Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells," Molecular Biology of the Cell, 2005, vol. 16, pp. 5719-5735.
Tesar et al., Nature, 2007, vol. 448, pp. 196-199.
Toyooka et al., Development, 2008, vol. 135, pp. 909-918.
Ueda et al., PLoS ONE 3, 2008, e2800.
Wernig et al., "c-Myc is Dispensable for Direct Reprogramming of Mouse Fibroblasts," Cell Stem Cell, 2008, 2, 10-12.
Xu et al., Nature 453, 338-44 (2008).
Ying et al, Nature, 2008, vol. 453, pp. 519-523.
Ying et al., Cell, 2003, vol. 115, pp. 281-292.
Yu et al., Science, 2007, vol. 318, pp. 1917-1920.
Xu et al., Nat. Biotechnol, 2002, vol. 20, pp. 1261-1264.
Zhou, Hongyan et al.; "Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins"; Cell Stem Cell, 4: 381-384 (2009).
Zhu et al., "Reprogramming of human primary somatic cells by OCT4 and chemical compounds," Cell Stem Cell, Dec. 3, 2010, vol. 7, No. 6, pp. 651-655.

(56) **References Cited**

OTHER PUBLICATIONS

Kim et al., Cell, 2009, vol. 136, pp. 411-419.
Office Action mailed Nov. 23, 2013, U.S. Appl. No. 12/933,391.
Office Action mailed May 17, 2013, U.S. Appl. No. 12/723,063.
Classen et al., "ROCK inhibition enhances the recovery and growth of cryopreserved human embryonic stem cells and human induced pluripotent stem cells," Molecular Reproduction and Developments, 2009, vol. 76, No. 8, pp. 722-732.
Hanna et al, "Treatment of Sickle Cell Anemia Mouse Model with iPS Cells Generated from Autologous Skin," Science, Dec. 21, 2007, vol. 318, pp. 1920-1922.
Shi "Induction of Pluripotent Stem Cells from Mouse Embryonic Fibroblasts by Oct4 and Klf4 with Small-Molecule Compounds," Cell Stem Cell, 2008, vol. 3, pp. 568-574.
U.S. Appl. No. 12/933,391, Final Office Action, Mailed Jul. 25, 2013.
Artyomov et al., PLoS Comput Biol 6, e1000785 (2010).
Brambrink et al., Cell Stem Cell 2, 151-9 (2008).
Christen et al., BMC Biol 8, 5 (2010).
Ernst et al., "gp130-mediated Signal Transduction in Embryonic Stem Cells Involves Activation of Jak and Ras/Mitogen-activated Protein Kinase Pathways," J. Biol. Chem., Nov. 22 1996, vol. 271, No. 47, pp. 30163-30143.
Graf et al., Nature 462(7273):587-594 (2009).
Han et al., Nat Cell Biol 13(1):66-71 (2011).
Hanna et al., Cell 133, 250-64.
Hanna et al., Nature 462, 595-601 (2009).
Hochedlinger et al., Development 136, 509-23 (2009).
Ieda et al., Cell 142, 375-86 (2010).
Jia et al., Nat Methods 7(3):197-199 (2010).
Kim et al., "Direct reprogramming of mouse fibroblasts to neural progenitors," Proc. Natl. Acad. Sci., USA, May 10, 2011, vol. 108, No. 9, pp. 7838-7843.
Kuzmenkin et al., FASEB J. 23, 4168-80 (2009).
Mikkelsen et al., Nature 454(7200):49-55 (2008).
Okada et al., Biochem Biophys Acta 1800, 956-63 (2010).
Okita et al., Nature 448, 313-317 (2007).
Schenke-Layland et al., Stem Cell 26, 1537-46 (2008).
Silva et al., Cell 138, 722-37 (2009).
Sridharan et al., Cell 136(2):364-377 (2009).
Stadtfeld et al., Cell Stem Cell 2, 230-40 (2008).
Stadtfeld et al., Nat Methods 7, 53-55 (2010).
Szabo et al., Nature 468(7323):521-526 (2010).
Takahashi et al., Nat Protoc 2, 3081-9 (2007).
Tighe et al., BMC 8:34 doi//:www.biomedcentral.com/1471-2121/8/34, printout pp. 1-17.
Vierbuchen et al., "Direct conversion of fibroblasts to functional neurons by defined factors," Nature, Feb. 25 2010, vol. 463, No. 7284, pp. 1035-1042.
Warren et al., Cell Stem Cell 7(5):618-630 (2010).
Wernig et al., Nat Biotechnol 26, 916-24 (2008).
Takahashi et al., Cell 126, 663-676 (2006).
Zhou et al., Nature 455(7213):627-632 (2008).
Feldman et al., "G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis," Nature Cell Biology, 2006, vol. 8(2), pp. 188-194.
Wu et al., "Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation," PNAS, 2007, vol. 104(32), pp. 13028-13033.
Aoi et al., "Generation of Pluripotent Stem Cells from Adult Mouse Liver and Stomach Cells," Sciencexpress, Feb. 2008, DOI 10.1126/science.1154884, 8 pages.
Lin et al., Nat Methods 6:805-808 (2009), Supplemental Information, 7 pages.
Noggle et al., "A Molecular Basis for Human Embryonic Stem Cell Pluripotency," Stem Cell Reviews and Reports, Jan. 2005, vol. 1(2), pp. 1550-8943; DOI: 10.1385/scr:1:2:111.
Stadtfeld et al., "Reprogramming of Pancreatic β Cells into Pluripotent Stem Cells," Curr. Biol., Jun. 2008, vol. 18(12): 890, doi: 10.1016/j.cub.2008.05.010.
Vallier et al., "Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells," Journal of Cell Science, Oct. 2005, vol. 118(19), pp. 4495-4509, DOI: 10.1111/J.1432-0436.2006.00143.X.
Li, Wenlin et al., "Generation of novel rat and human pluripotent stem cells by reprogramming and chemical approaches," Methods in Molecular Biology, Jan. 2010, vol. 636, pp. 293-300.
Xiong et al., "Histone deacetylase inhibitors DNA methyltransferase-3B messenger RNA stability and down-regulate de novo DNA methyltransferase activity in human endometrial cells," Cancer Res., Apr. 2005, vol. 65(7), pp. 2684-2689.
Zhou et al., "Conversion of Mouse Epiblast Stem Cells to an Earlier Pluripotency State by Small Molecules," Journal of Biological Chemistry, Sep. 2010, vol. 285(39), pp. 29676-29680; DOI: 10.1074/jbc.C110.150599.
Chen et al., "Self-renewal of embryonic stem cells by a small molecule," PNAS, 103(46):17266-17271, 2006.
Watanabe et al., "A ROCK inhibitor permits survival of dissociated human embryonic stem cells," Nature Biotechnology, 25(6):681-868, 2007.
Xu et al., "Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules," PNAS, 2010, vol. 107(8), pp. 8129-8134.
Egler et al., "Histone Deacetylase Inhibition and Blockade of the Glycolytic Pathway Synergistically Induce Glioblastoma Cell Death," Clin. Cancer Res., 2008, vol. 14(10), pp. 3132-3140.
Engel et al., "Allosteric activation of the protein kinase PDK1 with low molecular weight compounds," The EMBO Journal, 2006, vol. 25, pp. 5469-5480.
Gonzalez et al., "Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector," PNAS, Jun. 2009, vol. 106(22), pp. 8918-8922.
Hakelien et al., "Transient alteration of cell fate using a nuclear and cytoplasmic extract of an insulinoma cell line," BBRC, 2004 vol. 316, pp. 834-841.
Pesce et al., "Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation," Mechanisms of Development, 1998, vol. 71, pp. 89-98.
Wang et al., "The Immunophilin FKBP12 Functions as a Common Inhibitor of the TGFβ Family Type 1 Receptors," Cell, Aug. 1996, vol. 86, pp. 435-444.
Watanabe et al., "Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells," Oncogene, 2006, vol. 25, pp. 2697-2707.
Zhao et al., "Resorcylic Acid Lactones: Naturally Occurring Potent and Selective Inhibitors of MEK," The Journal of Antibiotics, Dec. 1999, vol. 52(12), pp. 1086-1094.
Condorelli, G. et al., "Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: Implications for myocardium regeneration," PNAS, vol. 98, No. 19, Sep. 11, 2001, pp. 10733-10738.
Dravida, S. et al., "The transdifferentiation potential of limbal fibroblast-like cells," Developmental Brain Research, vol. 160, No. 2, Dec. 7, 2005, pp. 239-251.
Efe, Jem A. et al., "Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy," Nature Cell Biology, vol. 13, No. 3, Mar. 1, 2011, pp. 215-222.
Kaji et al., "Virus free induction of pluripotency and subsequent excision of reprogramming factors," Nature, Apr. 2009, vol. 458(7239), pp. 771-775.
Kim et al., "Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors," 2008, Nature 454:646-651.
Loh et al., "Generation of induced pluripotent stem cells from human blood," Blood, May 2009, vol. 113(22), pp. 5476-5479.
Takei, Shunsuke et al., "Bone morphogenetic protein-4 promotes induction of cardiomyocytes from human embryonic stem cells in serum-based embryoid body development," AJP Heart and Circulatory Physiology, vol. 296, No. 6, Jun. 2009, pp. H1793-H1803.
Takeuchi, Jun K. et al., "Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors," Nature, vol. 459, No. 7247, Jun. 4, 2009, pp. 708-711.
Office Action mailed Sep. 25, 2015; U.S. Appl. No. 12/933,391.

(56) **References Cited**

OTHER PUBLICATIONS

Kim et al., Cell Stem Cell 4(6):472-476, 2009.
Okita et al., Science 322:949-953, 2008.
Stadtfeld et al., Science 322:945-949.
Tojo et al., Cancer Sci 96(11):791-800, 2005.
Tada et al., Current Biology 11:1553-1558, 2001.
Hochedlinger and Jaenisch, Nature 441:1031-1067, 2006.
Yamanaka, Cell Stem Cell 1:39-49, 2007.

* cited by examiner

FIG. 1

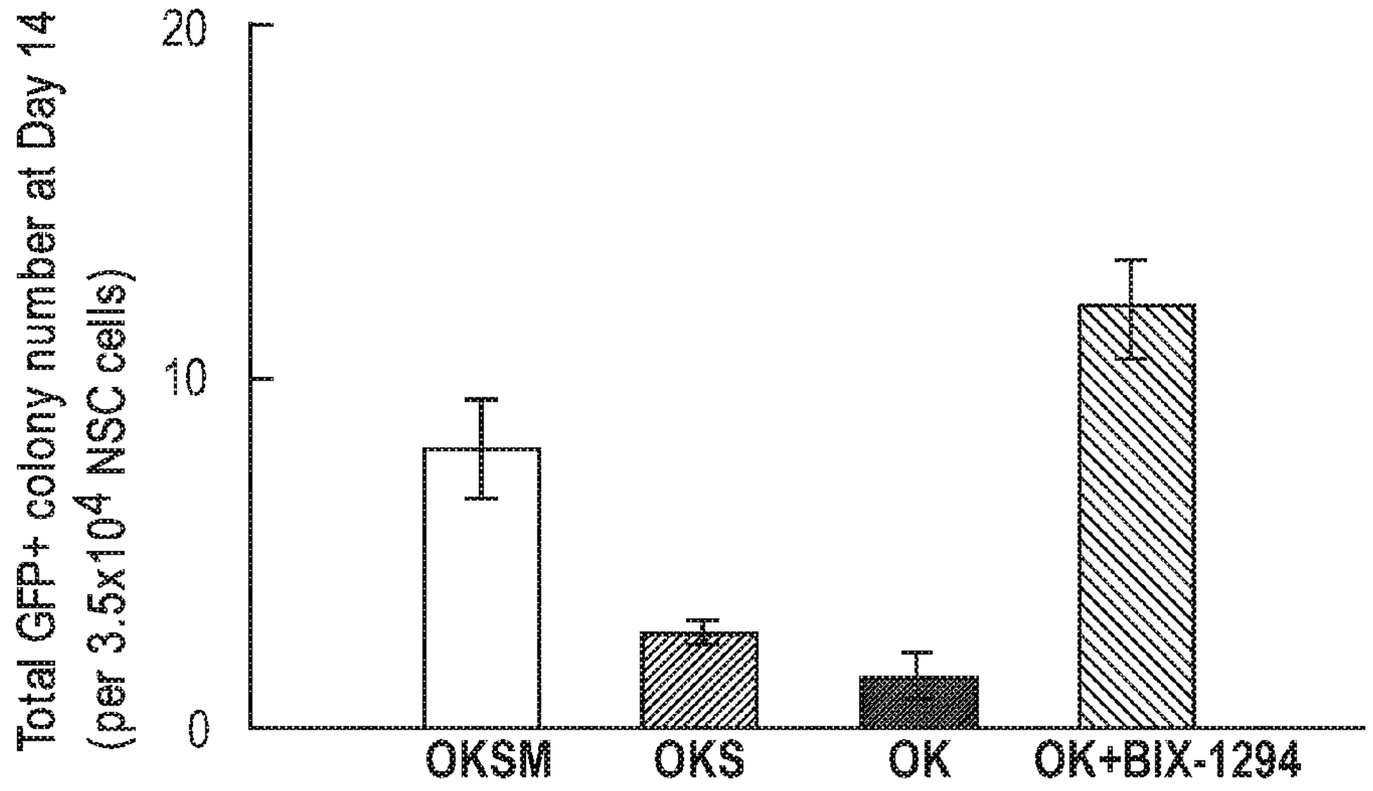

Total GFP+ colony number at Day 14
(per 3.5x10^4 NSC cells)
20
10
0
OKSM
OKS
OK
OK+BIX-1294

FIG. 2

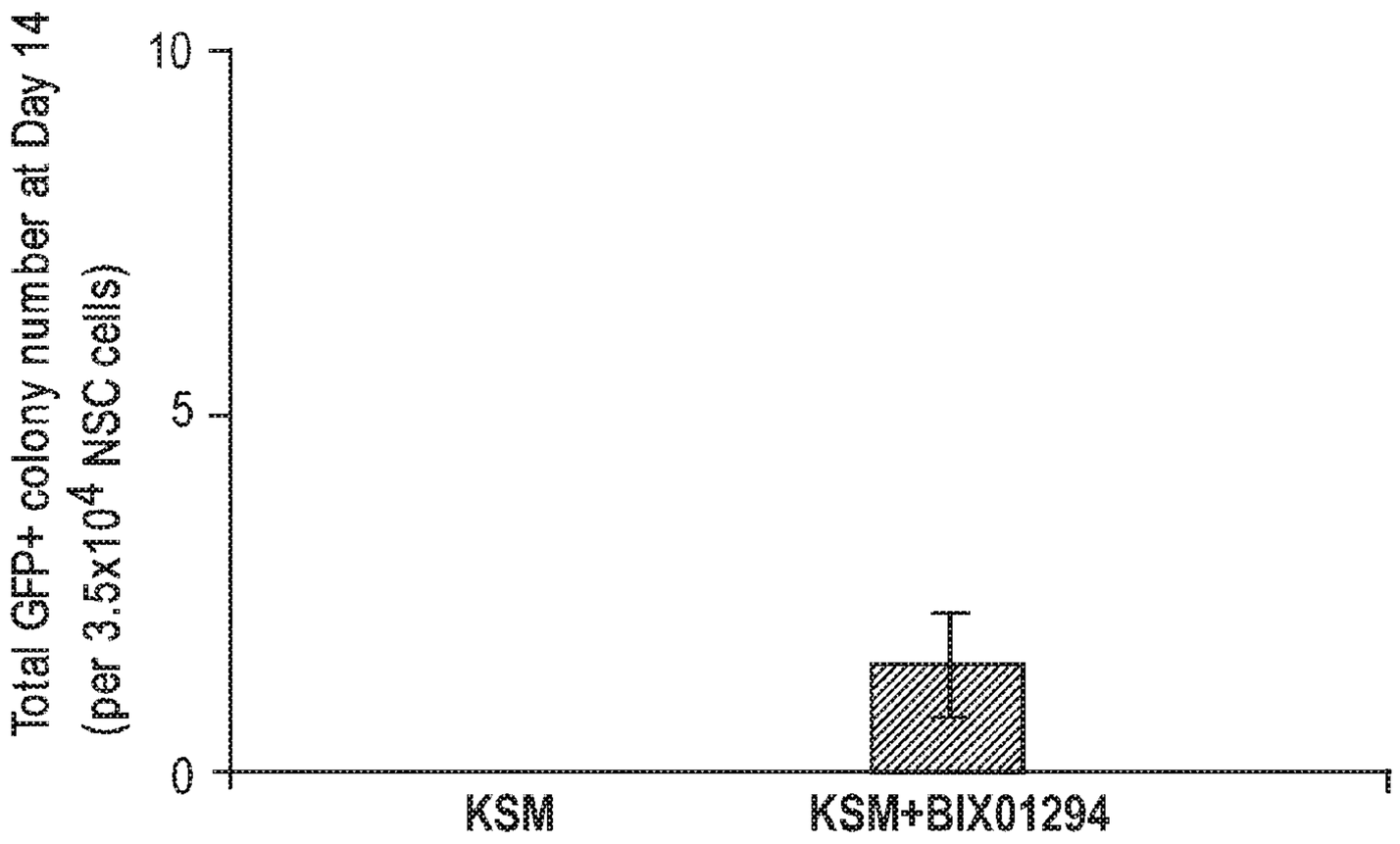

Total GFP+ colony number at Day 14
(per 3.5x10^4 NSC cells)
10
5
0
KSM
KSM+BIX01294

FIG. 3

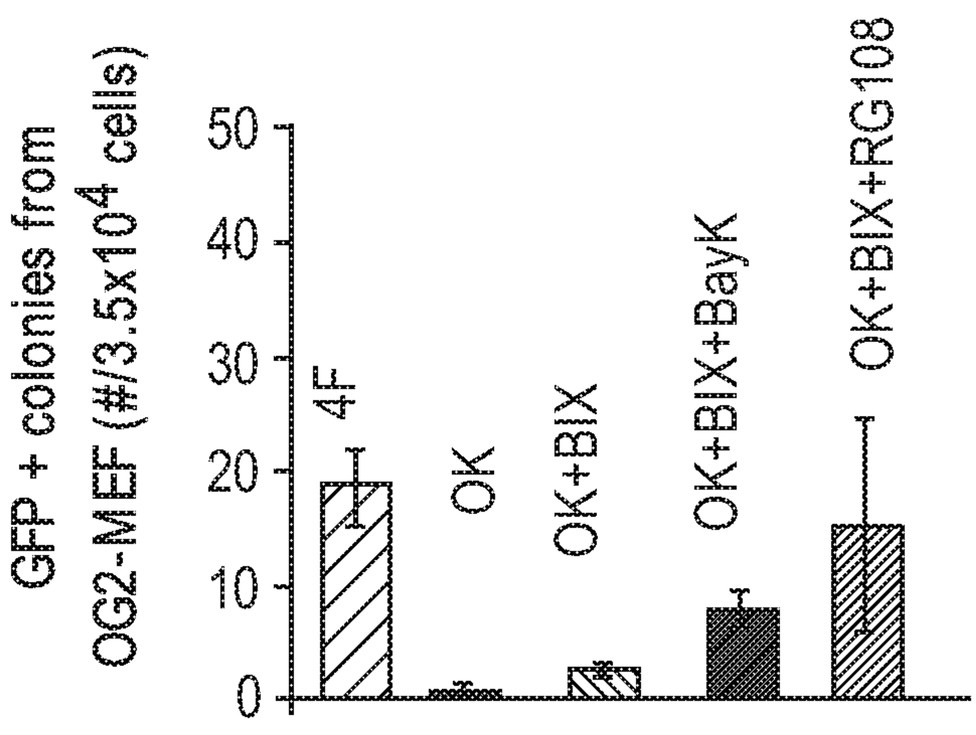

GFP + colonies from
OG2-MEF (#/3.5x10^4 cells)
0
10
20
30
40
50
4F
OK
OK+BIX
OK+BIX+BayK
OK+BIX+RG108

FIG. 4A

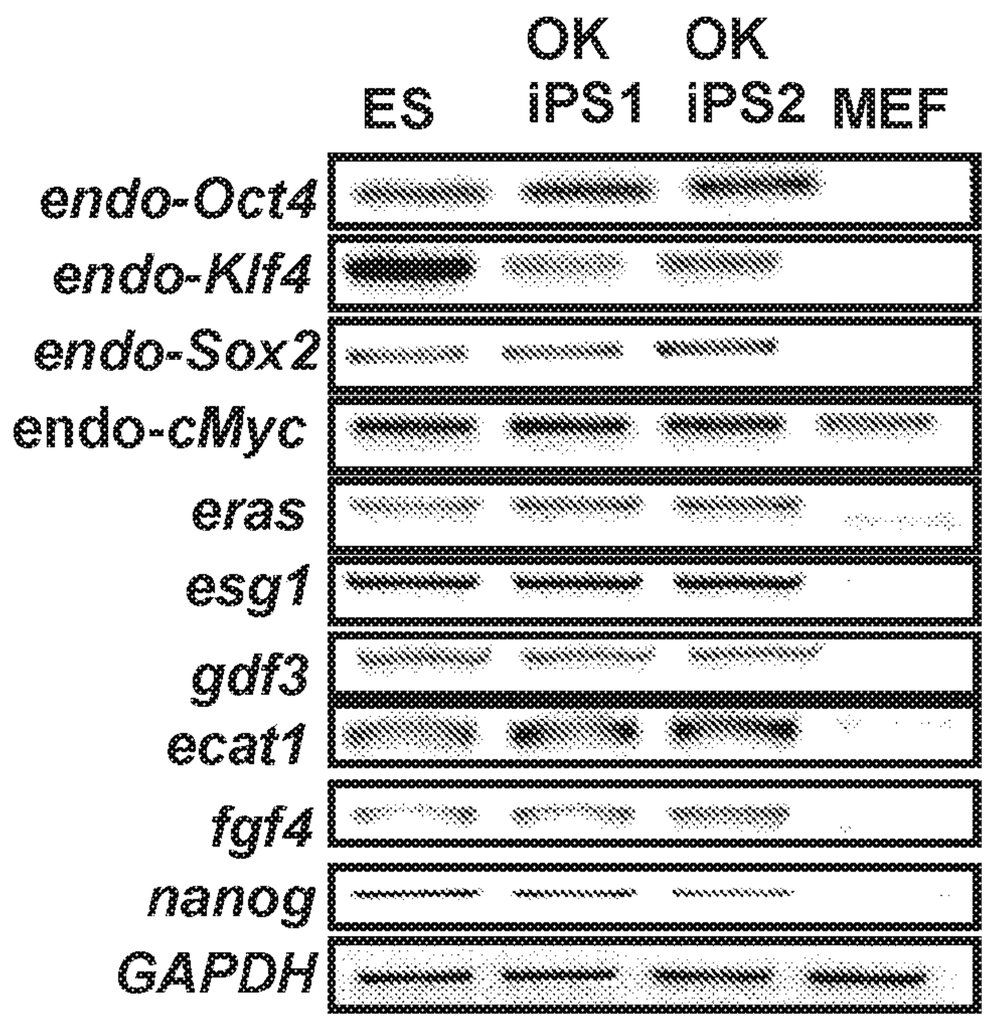

ES
OK iPS1
OK iPS2
MEF
endo-Oct4
endo-Klf4
endo-Sox2
endo-cMyc
eras
esg1
gdf3
ecat1
fgf4
nanog
GAPDH

FIG. 4B

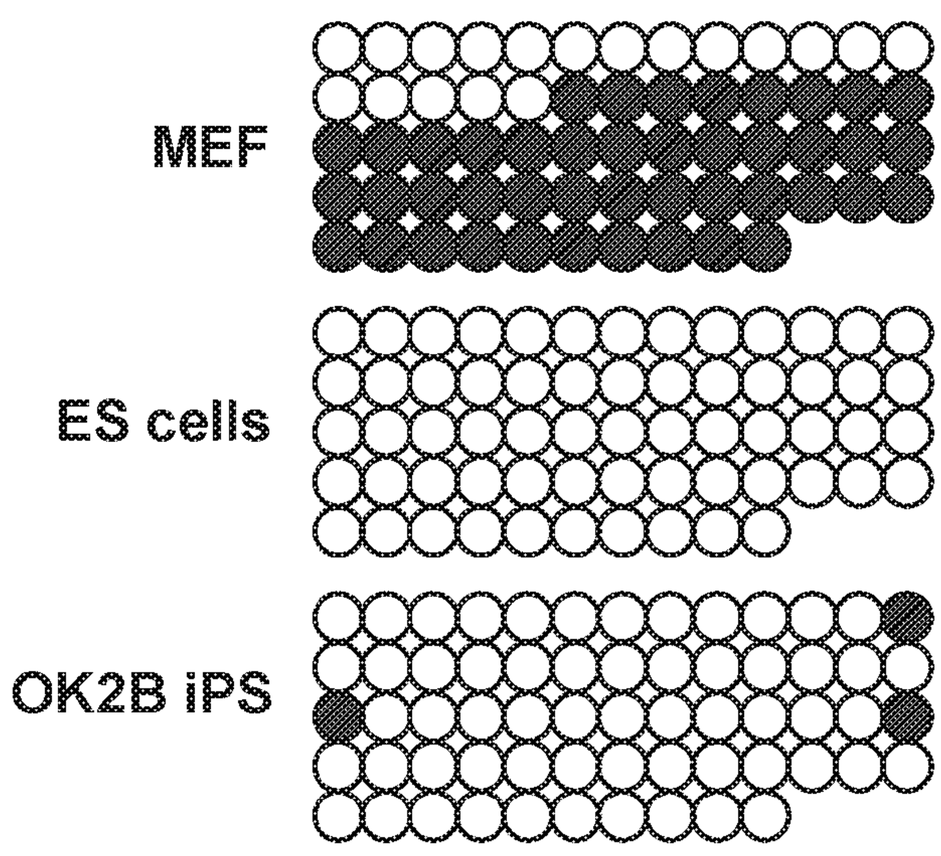
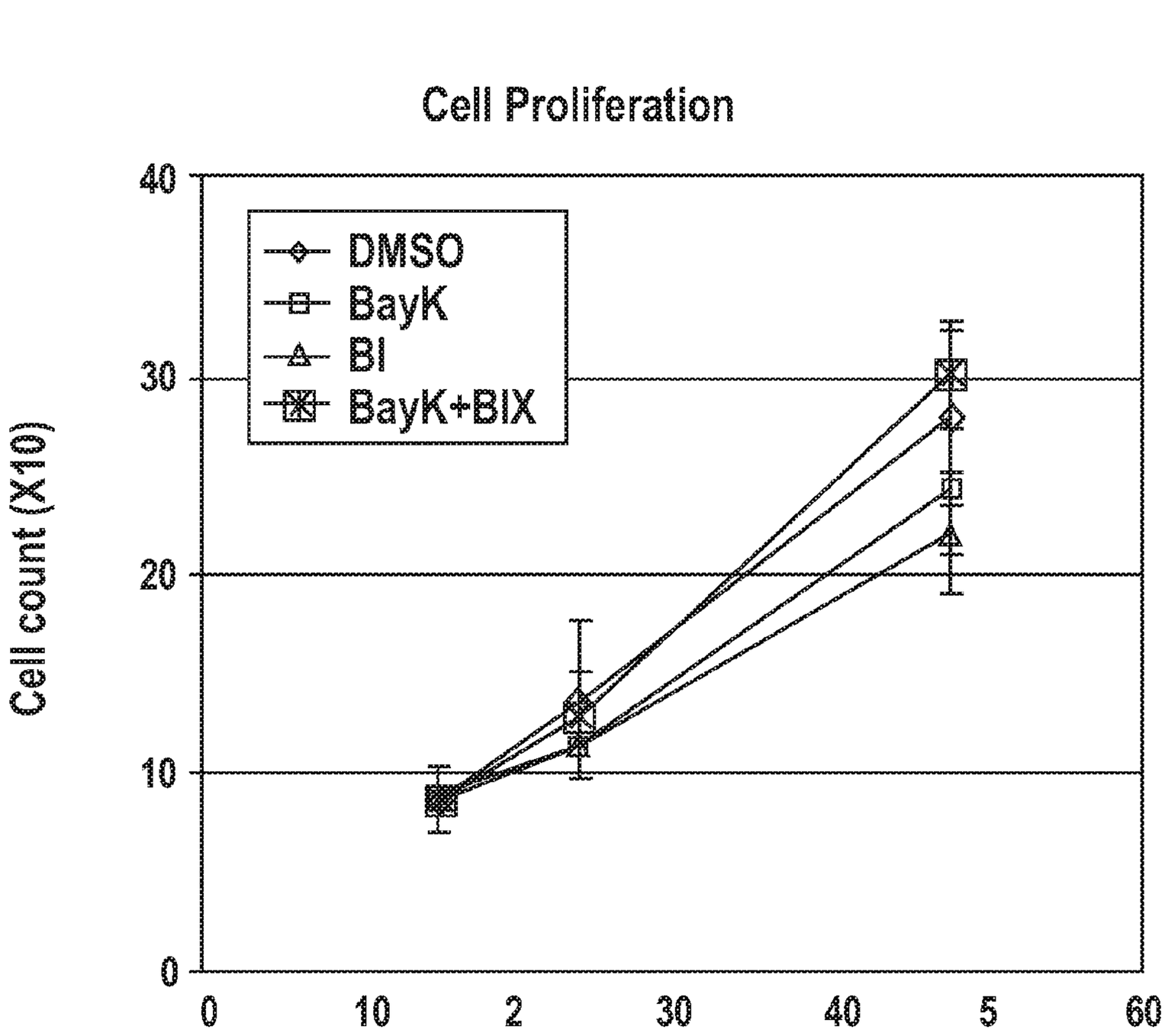

MEF
ES cells
OK2B iPS

FIG. 5


Cell Proliferation
Cell count (X'10)
40
30
20
10
0
DMSO
BayK
BI
BayK+BIX
0
10
2
30
40
5
60
Hours after

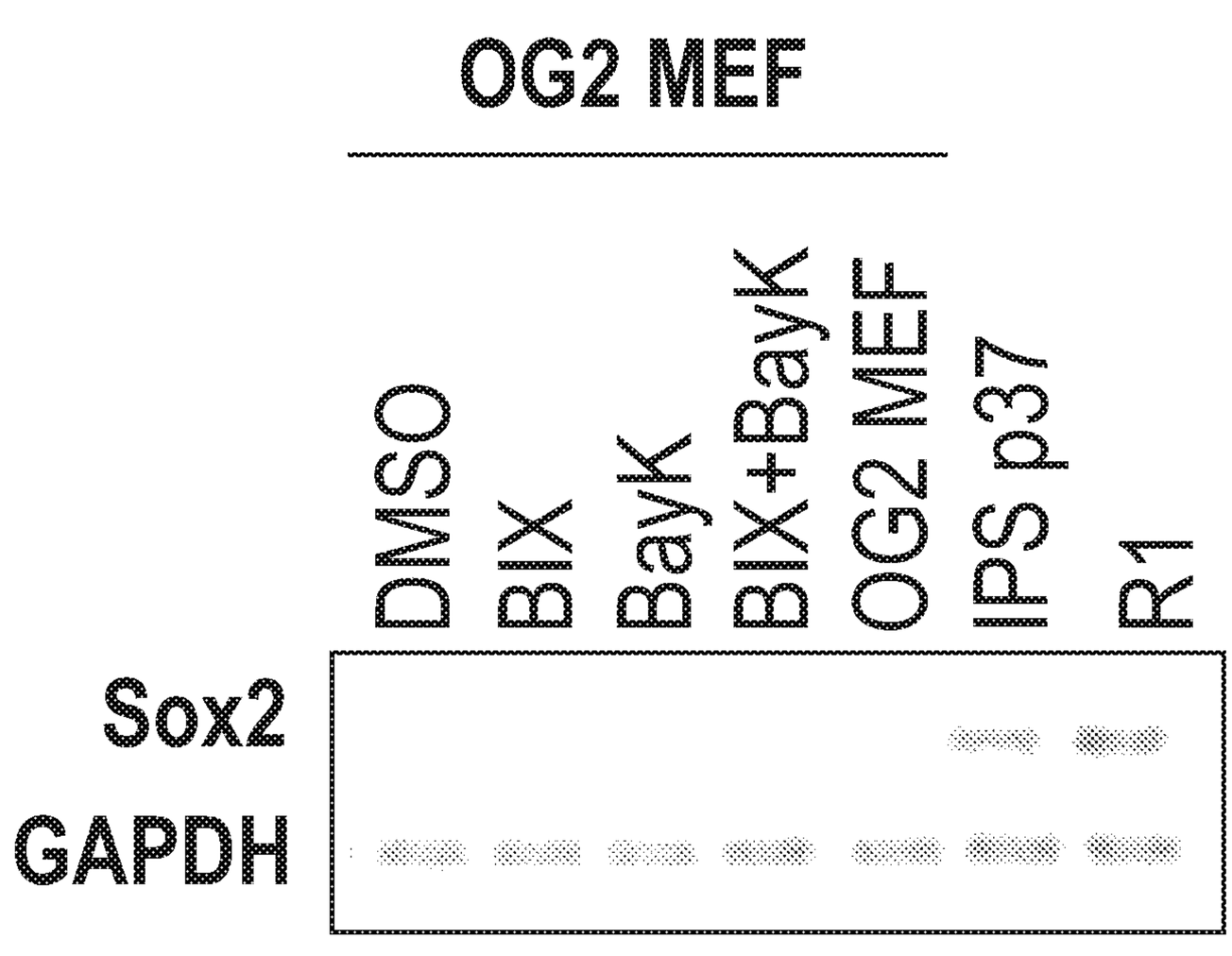


FIG. 6

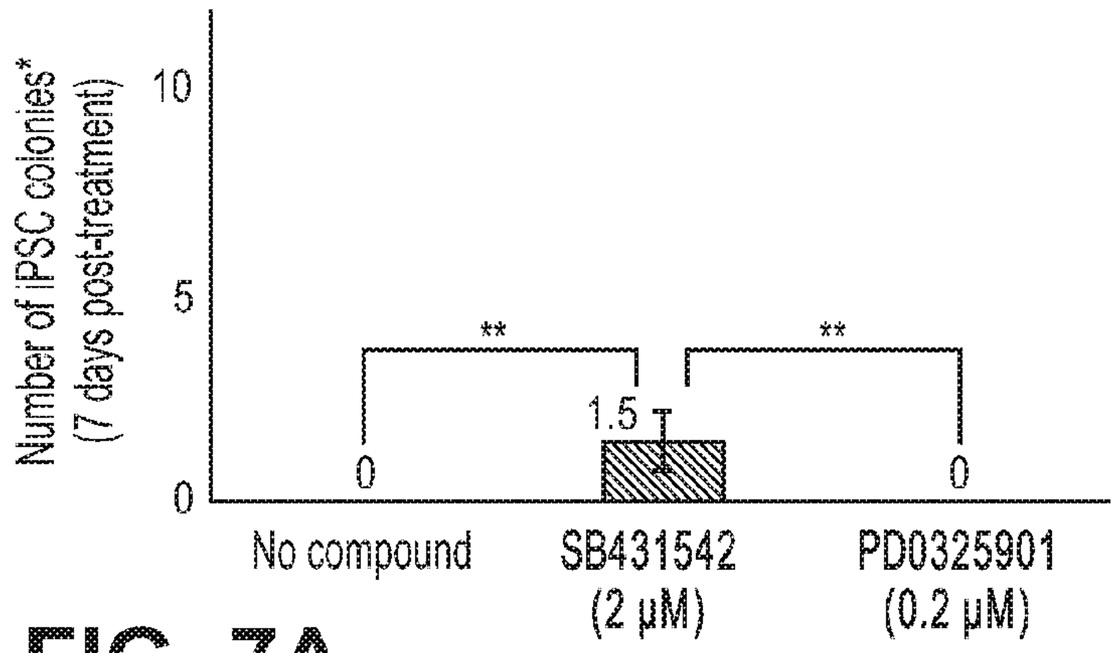


FIG. 7A

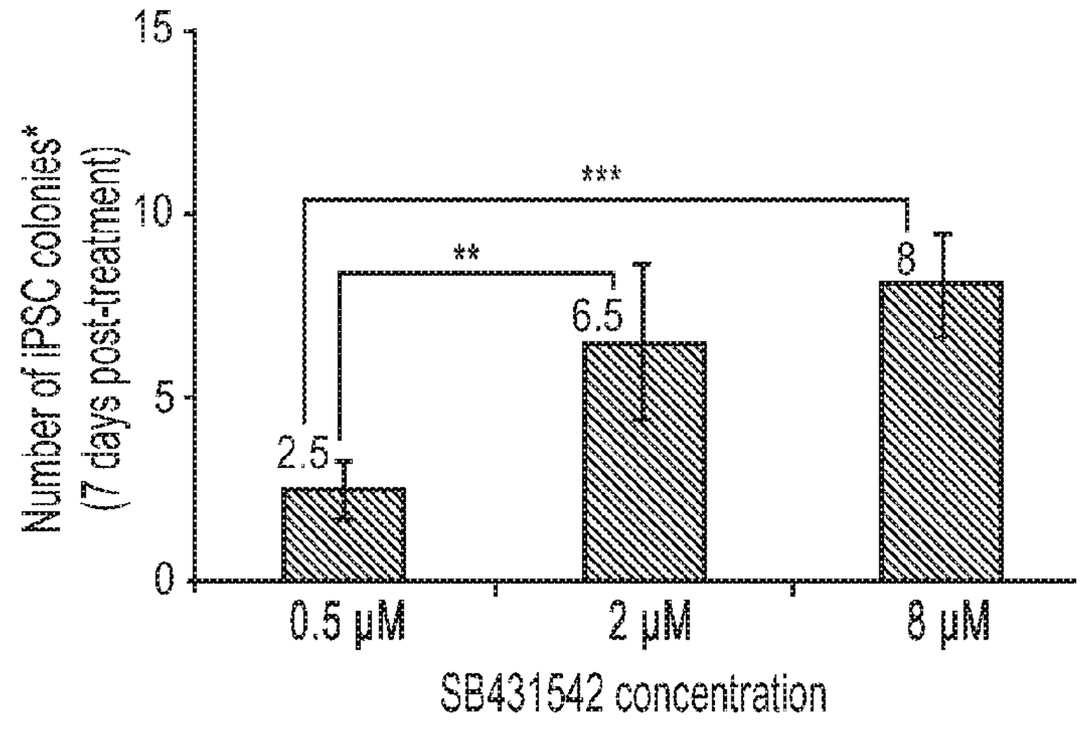


FIG. 7B

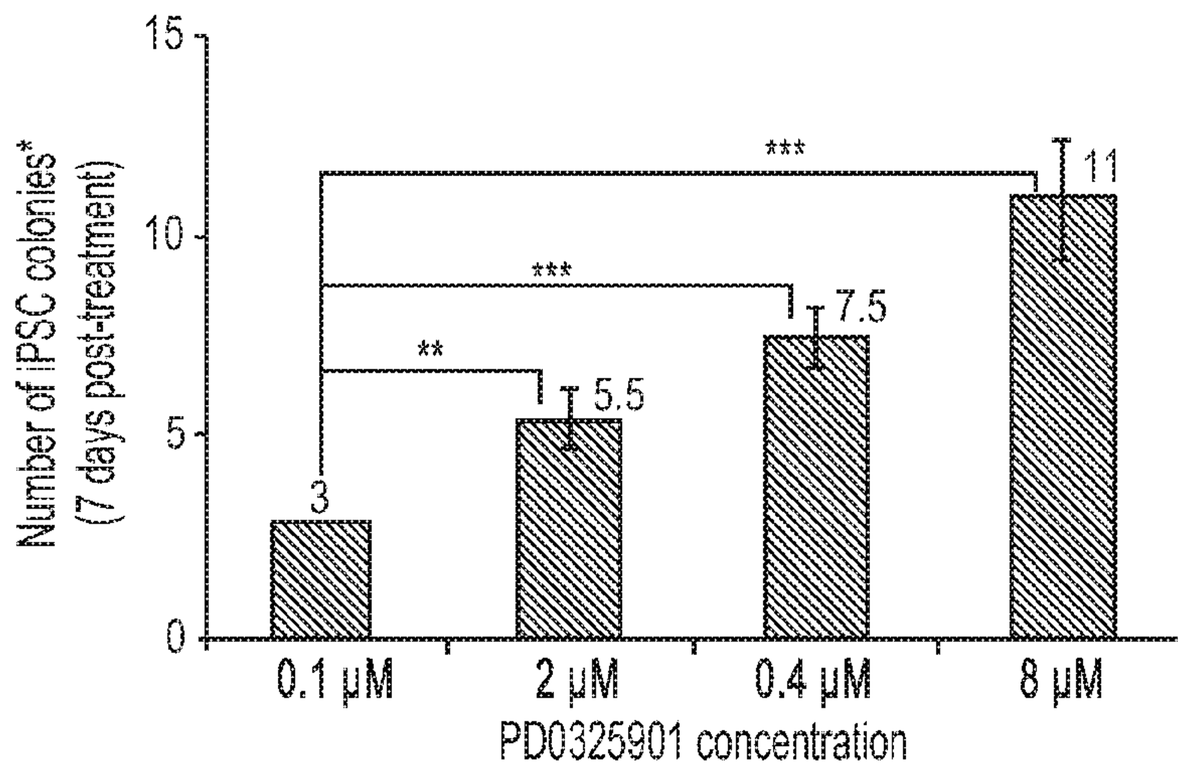


FIG. 7C

FIG. 8A

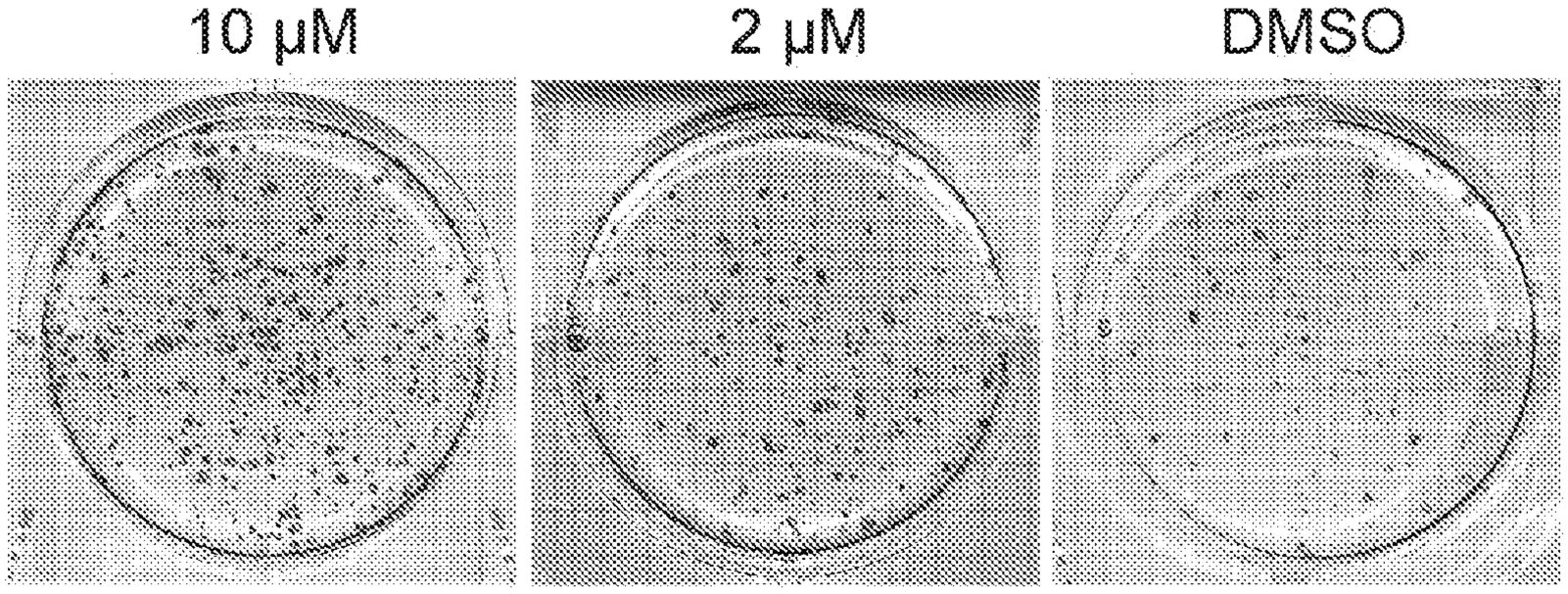

10 μM
2 μM
DMSO

FIG. 8B

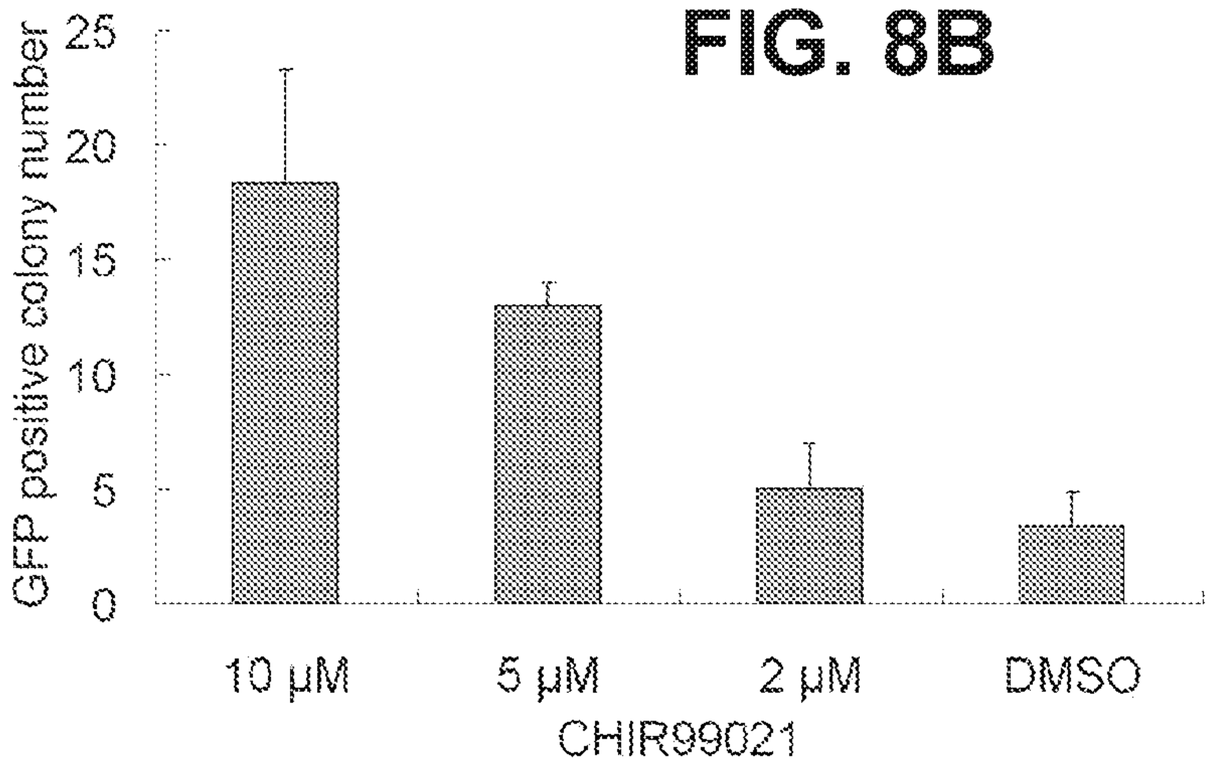

GFP positive colony number
25
20
15
10
5
0
10 μM
5 μM
2 μM
DMSO
CHIR99021

FIG. 9A

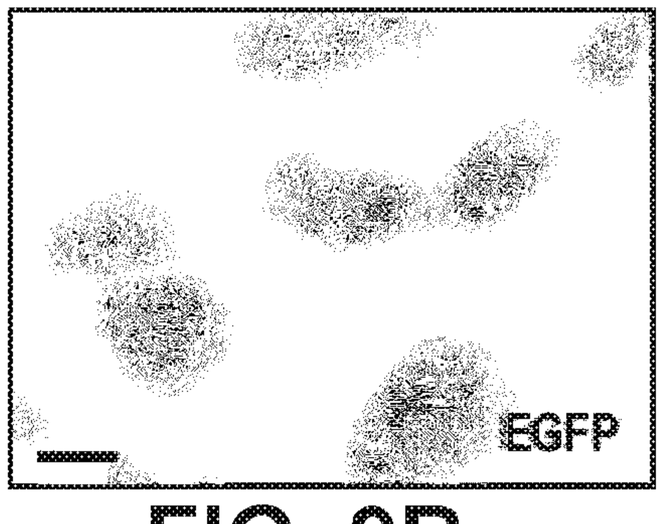


FIG. 9B

FIG. 9C

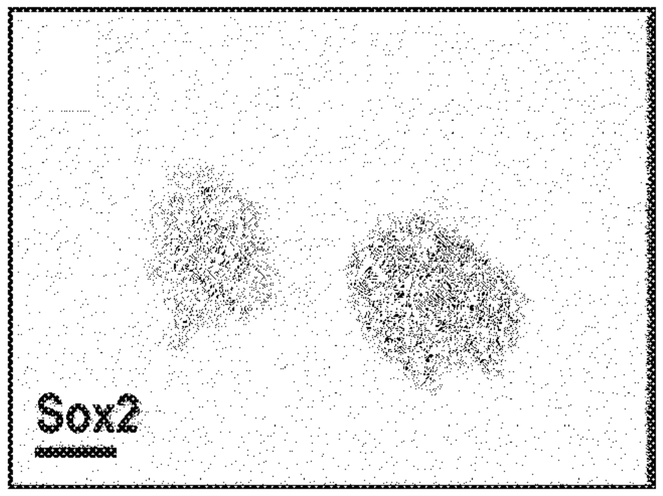


FIG. 9D

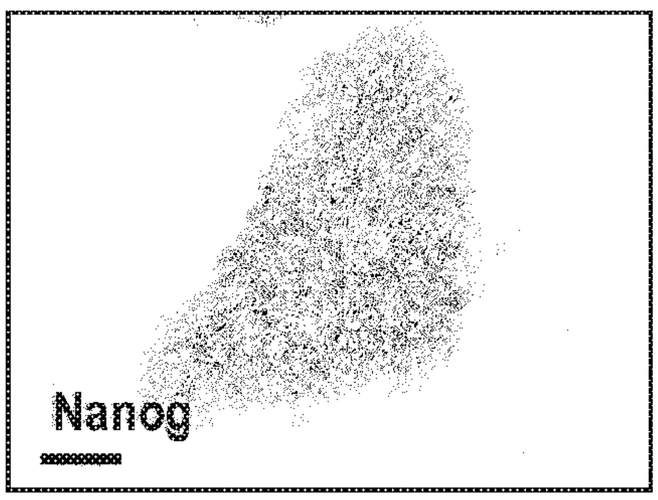


FIG. 9E

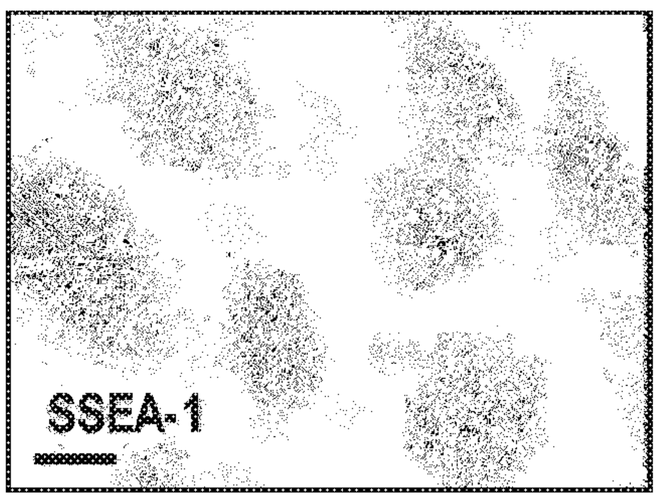


FIG. 9F

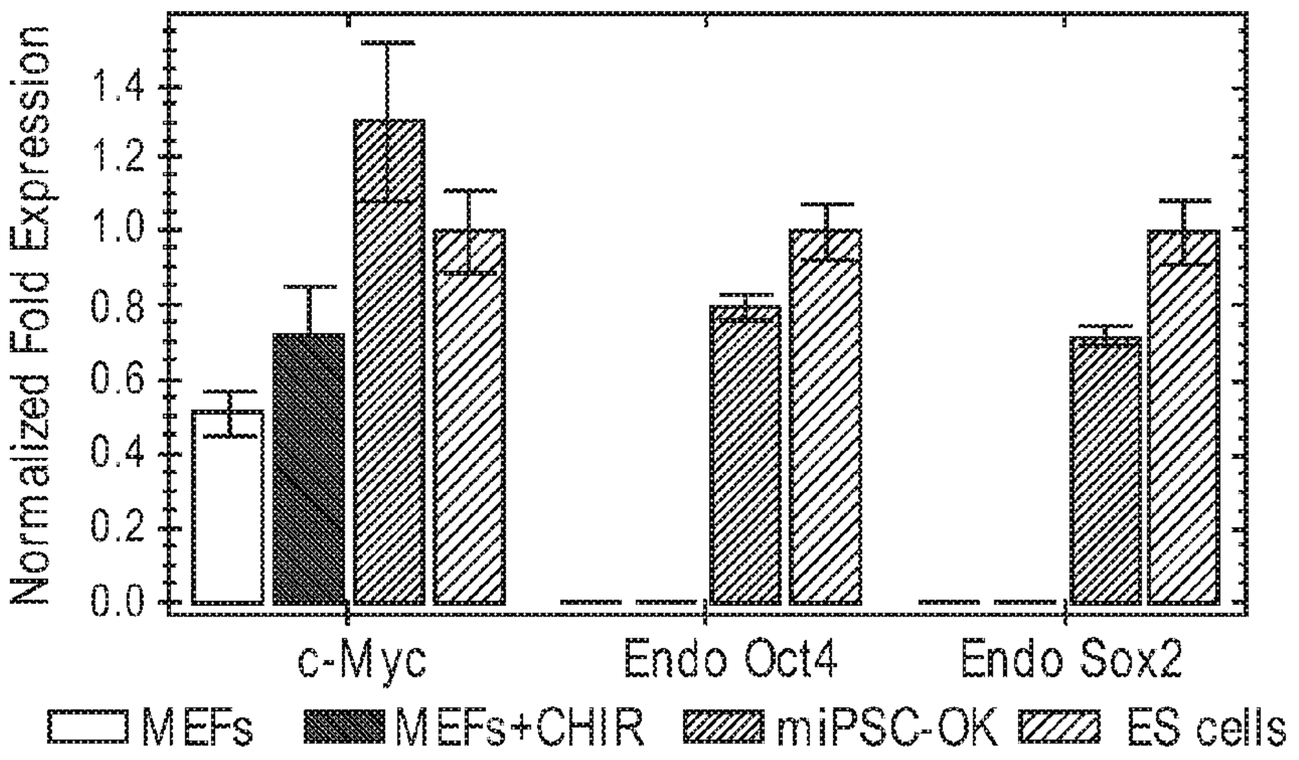


FIG. 9G

FIG. 10A

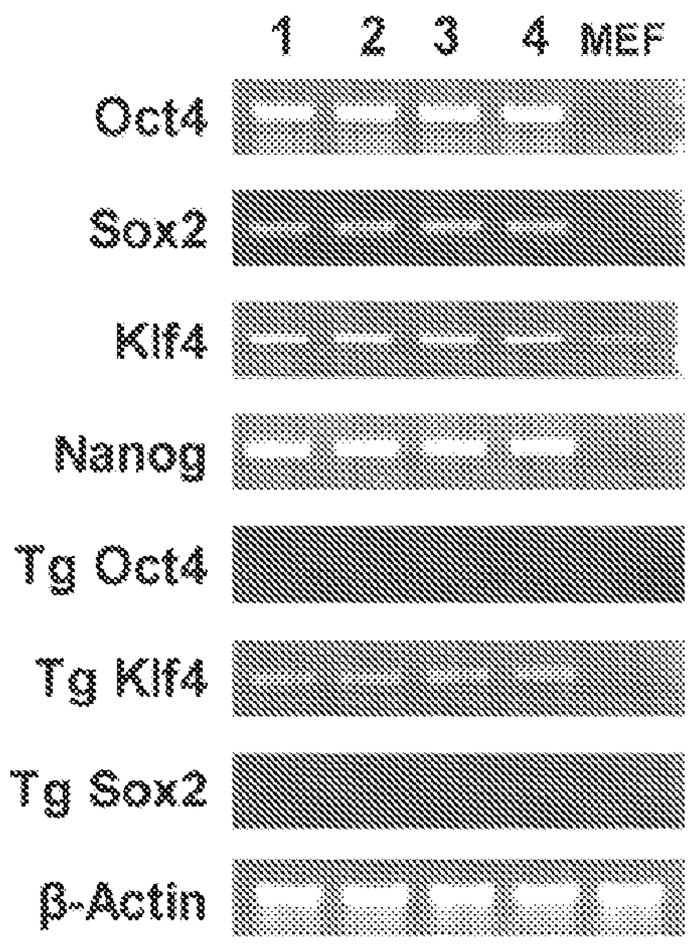


FIG. 10B

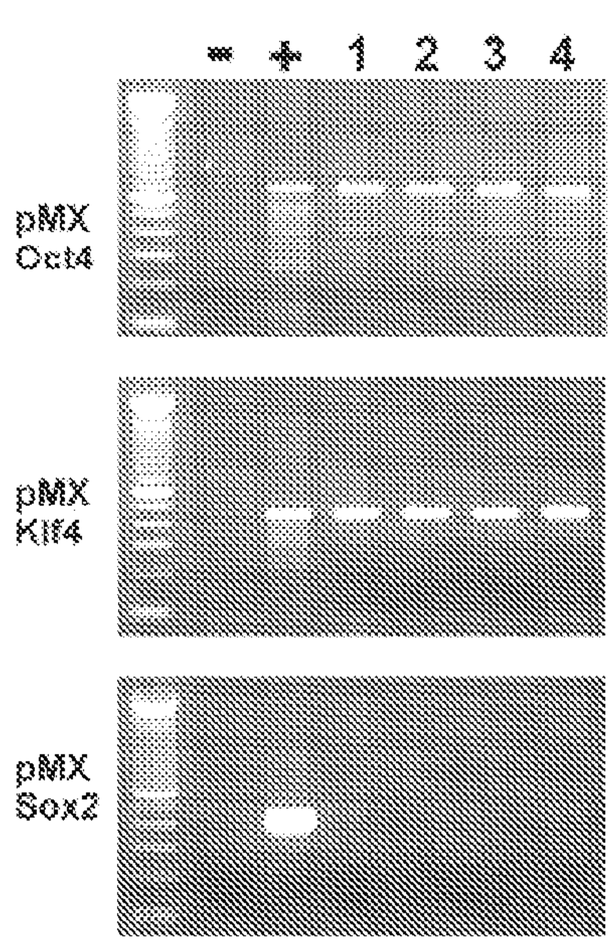


FIG. 10C

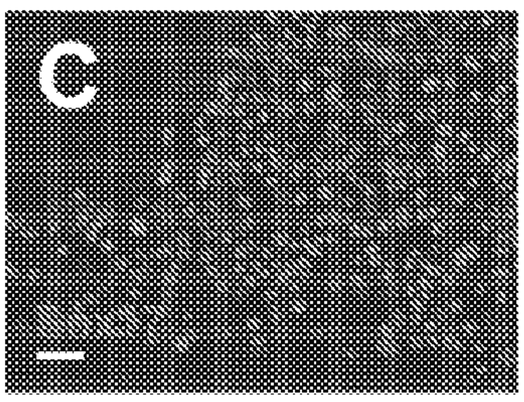


FIG. 10D

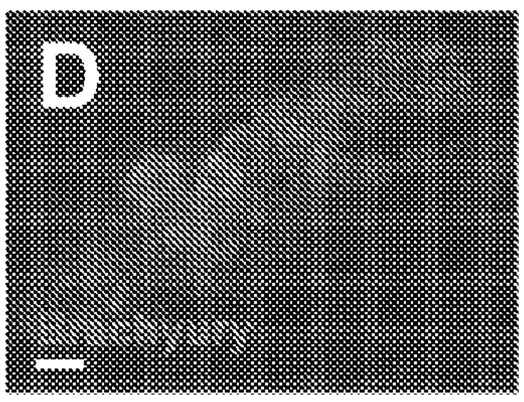


FIG. 10E

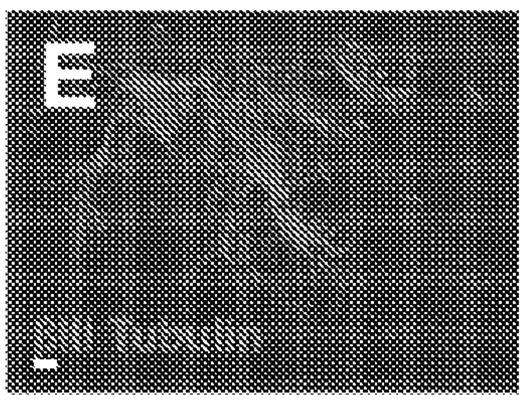


FIG. 10F

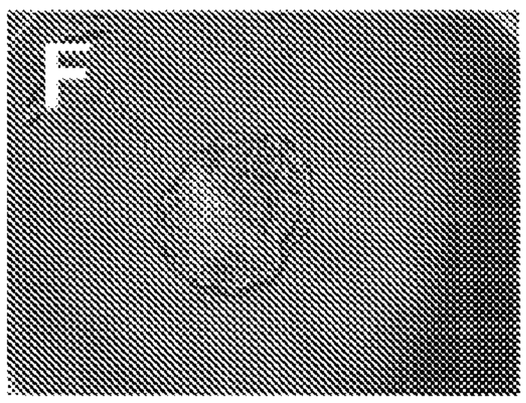


FIG. 10G

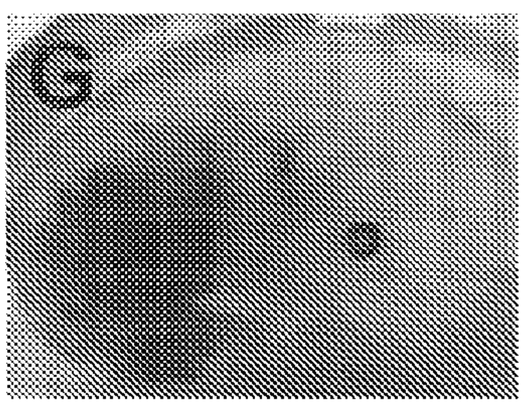
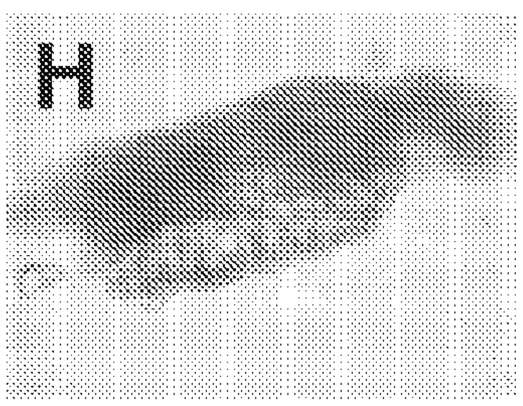


FIG. 10H



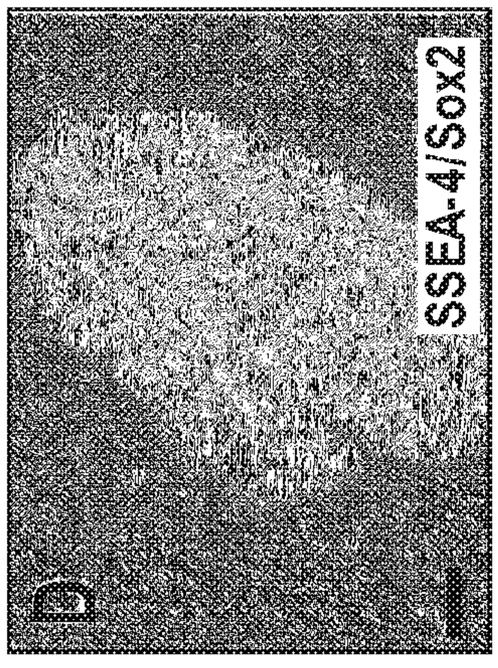


FIG. 11D

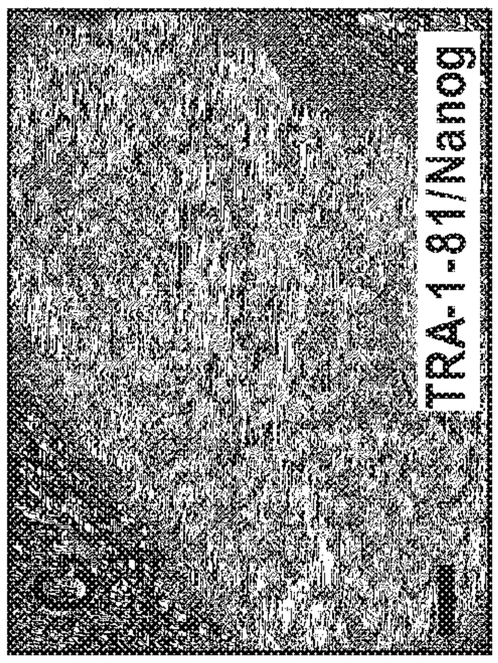


FIG. 11C

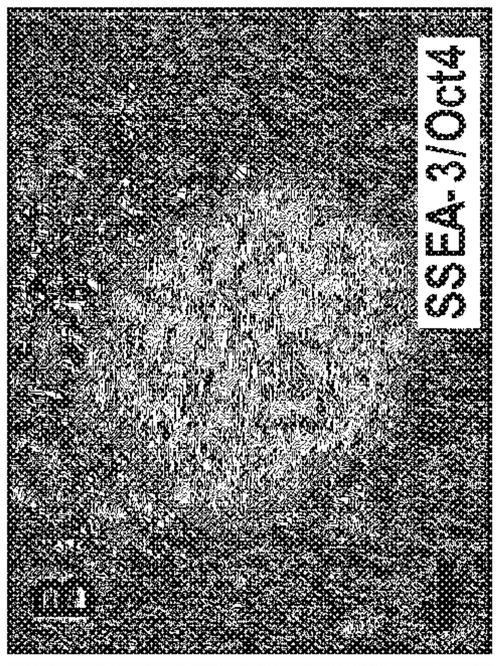


FIG. 11B

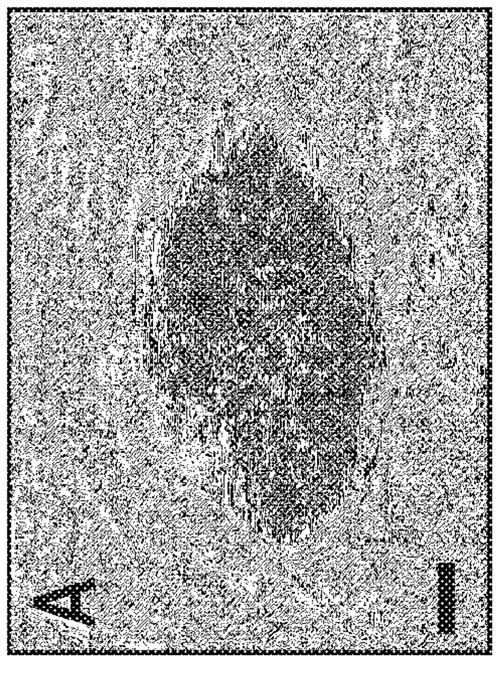


FIG. 11A

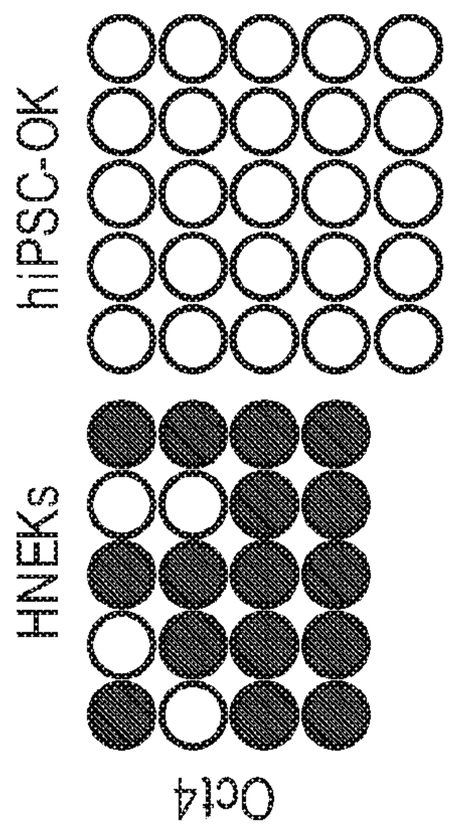


FIG. 11F

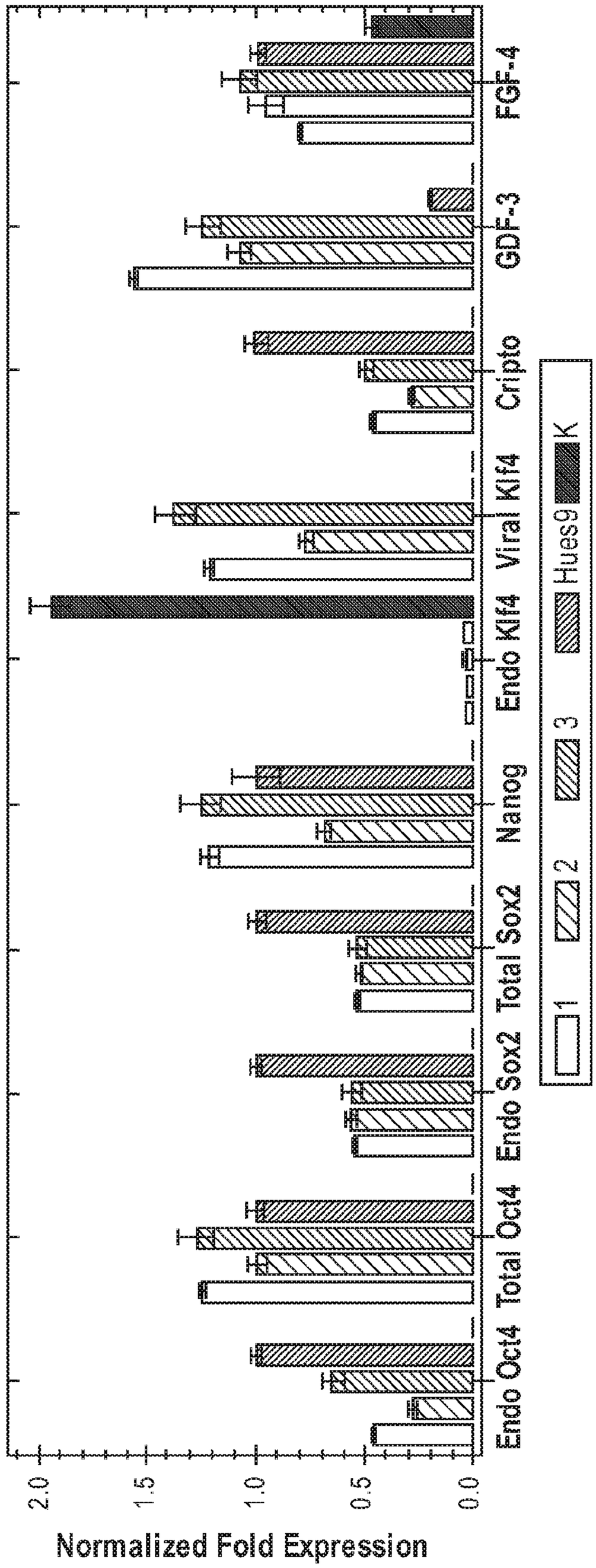

Endo Oct4
Total Oct4
Endo Sox2
Total Sox2
Nanog
Endo Klf4
Viral Klf4
Cripto
pto
GDF-3
FGF-4
1
2
3
Hues9
K
0.0
0.5
1.0
1.5
2.0
Normalized Fold Expression

FIG. 1E

FIG. 12

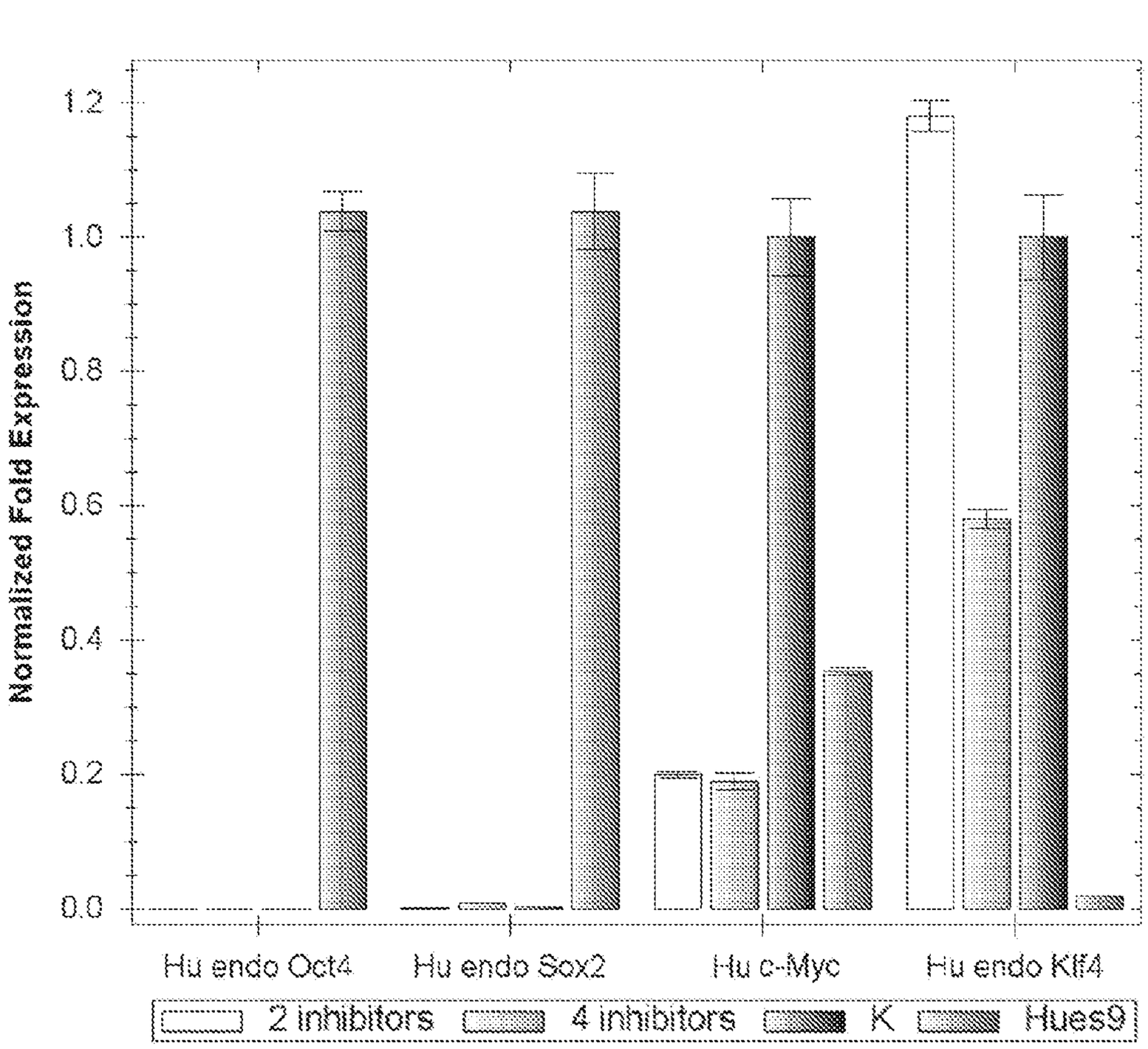

Normalized Fold Expression
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Hu endo Oct4
Hu endo Sox2
Hu c-Myc
Hu endo Klf4
2 inhibitors
4 inhibitors
K
Hues9

FIG. 13A

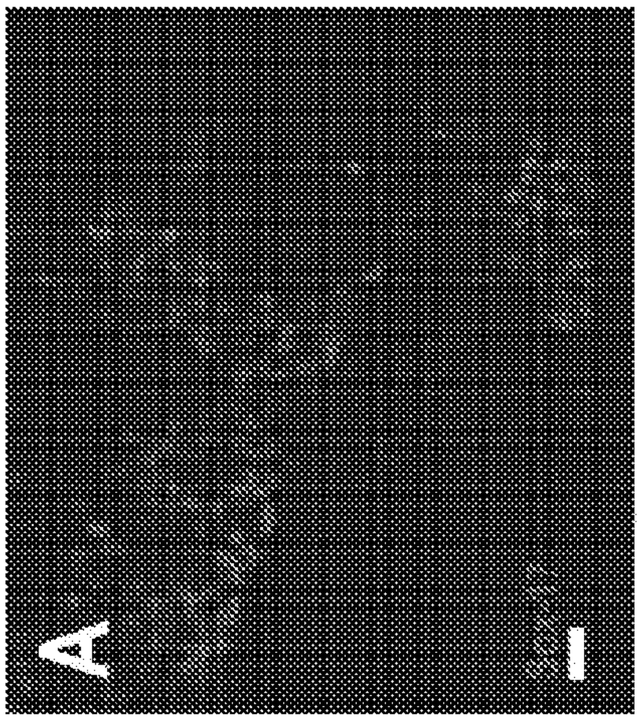


FIG. 13B

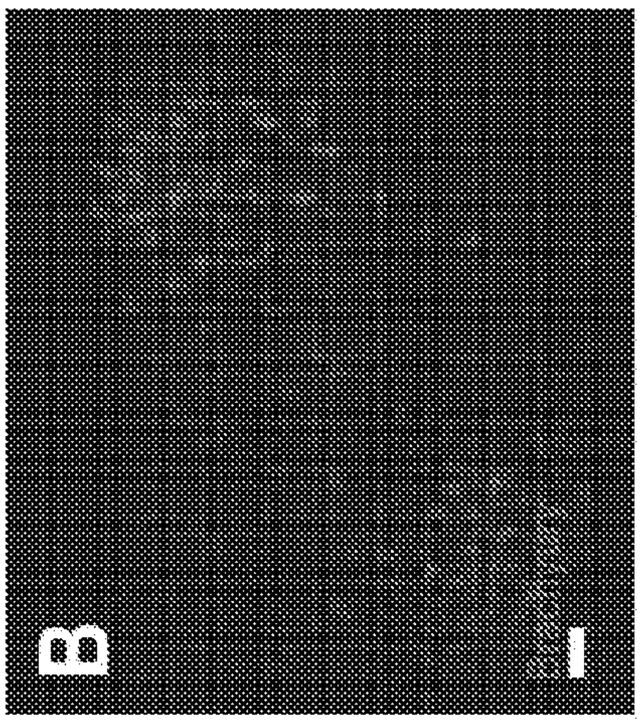


FIG. 13C

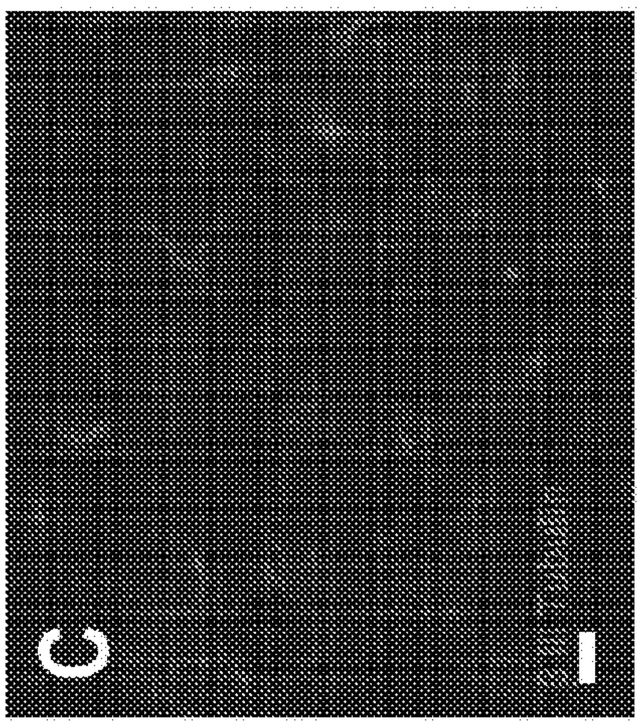


FIG. 13D

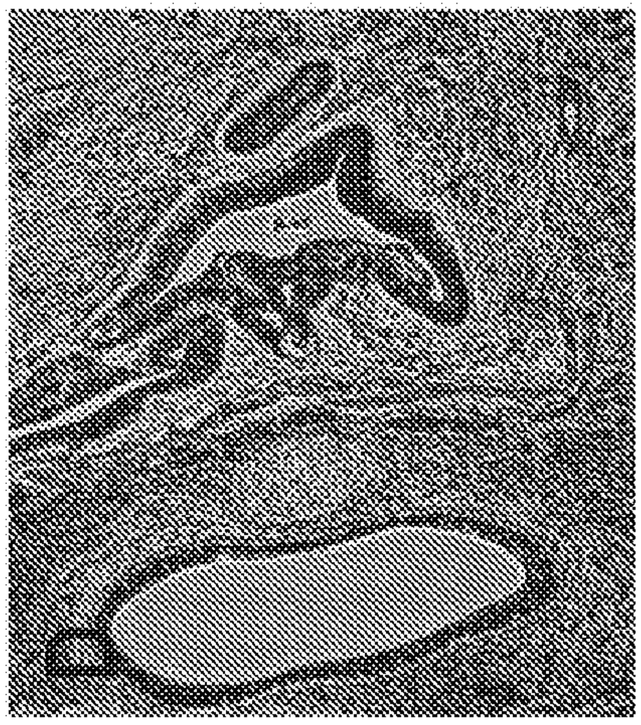


FIG. 13E

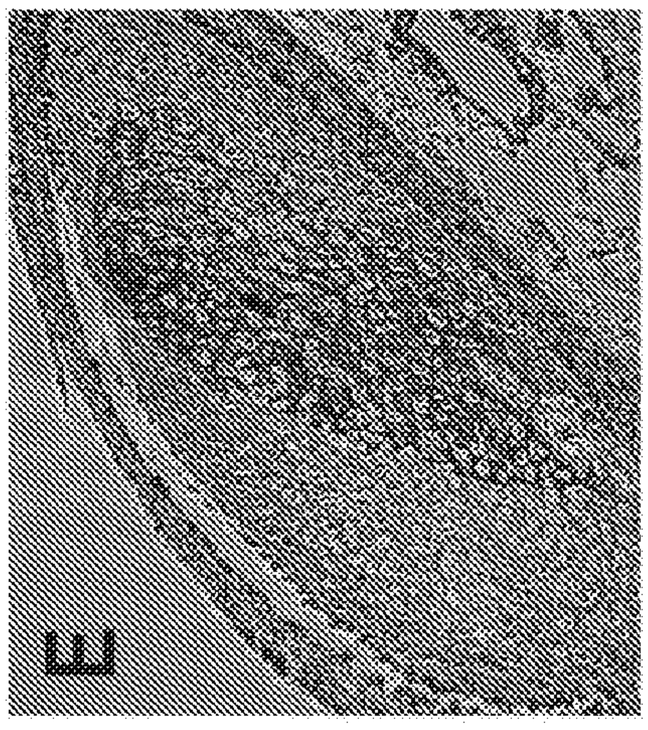
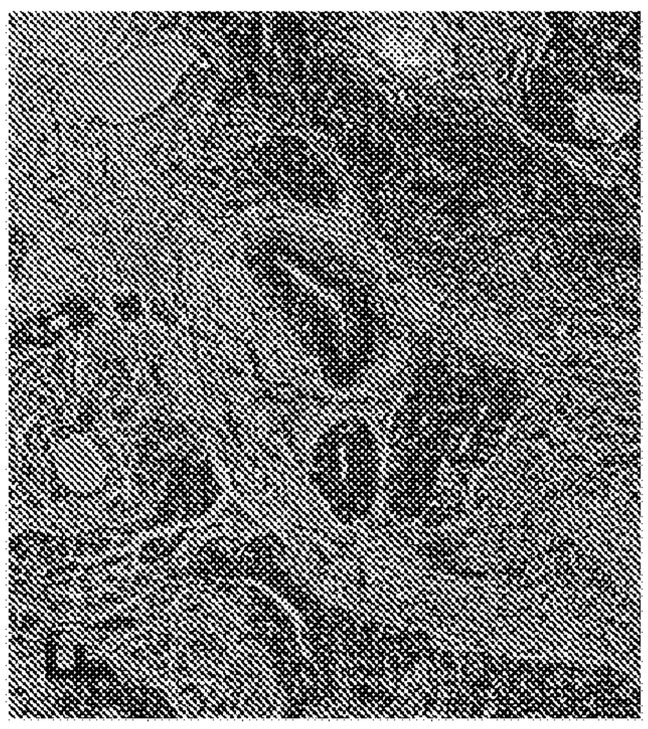


FIG. 13F



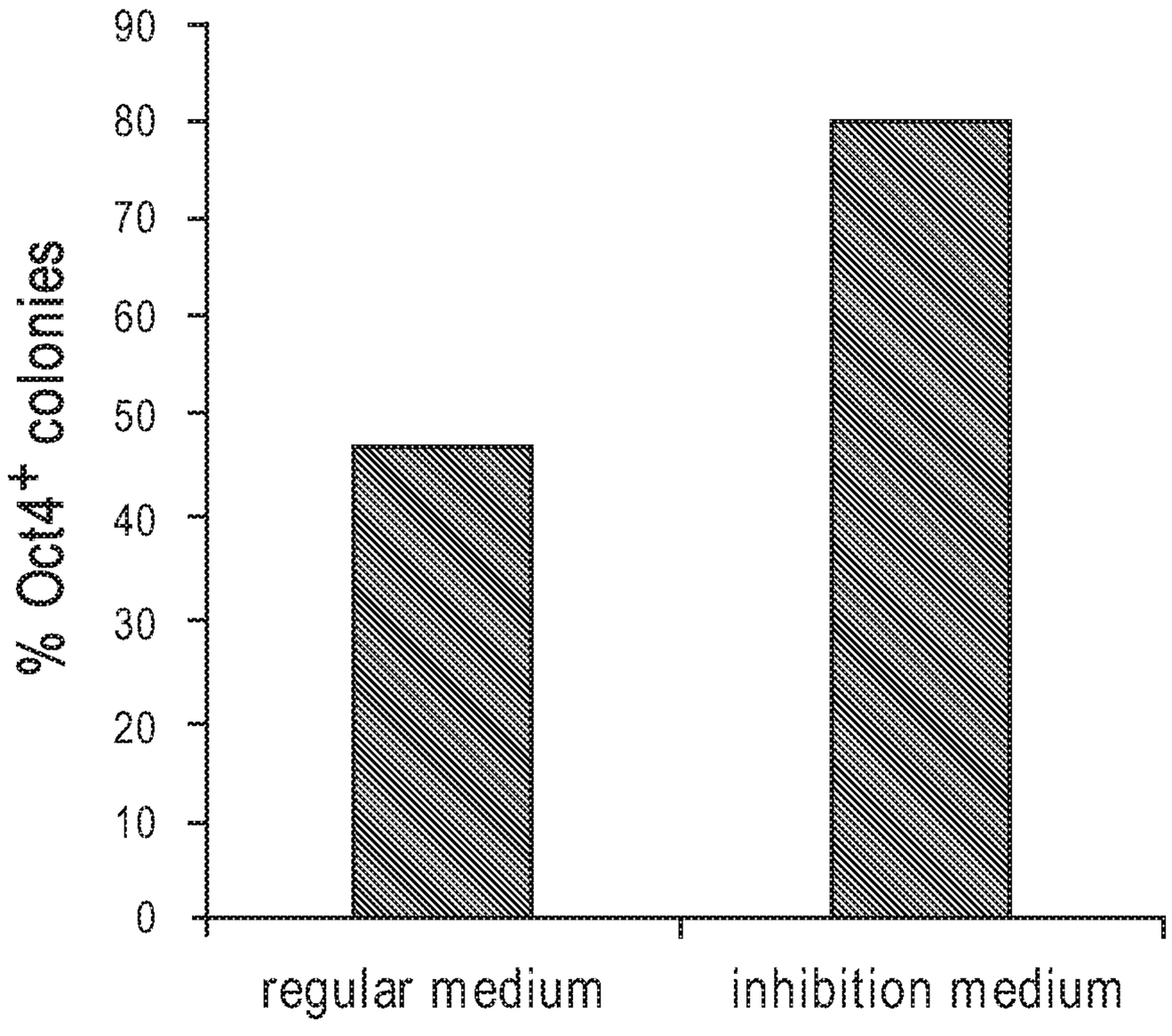

% Oct4+ colonies
0
10
20
30
40
50
60
70
80
90
regular medium
inhibition medium

FIG. 14

CHEMICAL APPROACHES FOR GENERATION OF INDUCED PLURIPOTENT STEM CELLS

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/753,386, filed Apr. 2, 2010, issued as U.S. Pat. No. 9,394,524, which is a continuation-in-part of PCT/US2009/037429 (published as WO2009/117439) filed Mar. 17, 2009 entitled "Combined Chemical and Genetic Approaches for Generation of Induced Pluripotent Stem Cells" which claims priority to U.S. Provisional Patent Application No. 61/069,956, filed Mar. 17, 2008, and to U.S. Provisional Patent Application No. 61/197,986, filed Oct. 31, 2008, the entire content of each of which is incorporated herein by reference for all purposes.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

The Sequence Listing written in file 077103-002550US-1012016_SequenceListing.txt created on Jun. 9, 2016, 3,213 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

Stem cells are often classified as totipotent or pluripotent. A totipotent stem cell has differentiation potential which is total: it gives rise to all the different types of cells in the body. A fertilized egg cell is an example of a totipotent stem cell. Pluripotent stem cells can give rise to any cell type in the body derived from the three main germ cell layers or an embryo itself.

Pluripotent stem cells, such as embryonic stem cells (ESCs), proliferate rapidly while maintaining pluripotency, namely, the ability to differentiate into various types of cells. Embryonic stem cells are promising donor sources for cell transplantation therapies. However, human ESCs are also associated with ethical issues regarding the use of human embryos and rejection reactions after allogenic transplantation. It may be possible to overcome these issues by generating pluripotent stem cells directly from a patient's somatic cells. That somatic cell nuclei acquire an embryonic stem-like status by fusion with ESCs suggests the existence of 'pluripotency-inducing' factors. Previous studies have recently shown that retrovirus-mediated transfection with four transcription factors (Oct-3/4, Sox2, KLF4 and c-Myc), which are highly expressed in ESCs, into mouse fibroblasts has resulted in generation of induced pluripotent stem (iPS) cells. See, Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663-676 (2006); Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature* 448, 313-317 (2007); Wernig, M. et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448, 318-324 (2007); Maherali, N. et al. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* 1, 55-70 (2007); Meissner, A., Wernig, M. & Jaenisch, R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nature Biotechnol.* 25, 1177-1181 (2007); Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861-872 (2007); Yu, J. et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917-1920 (2007); Nakagawa, M. et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts *Nature Biotechnol.* 26, 101-106 (2007); Wernig, M., Meissner, A., Cassady, J. P. & Jaenisch, R. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* 2, 10-12 (2008). iPS cells are similar to ESCs in morphology, proliferation, and pluripotency, judged by teratoma formation and chimaera contribution.

A recent breakthrough of using defined genetic manipulation, i.e. viral transduction of few genes highly and/or specifically expressed in mouse or human embryonic stem (ES) cells, in reprogramming both mouse and human somatic cells to induced pluripotent stem (iPS) cells has opened up tremendous opportunities to generate patient-specific stem cells for various applications (e.g. cell-based therapy or drug discovery) without the controversies associated with the conventional human ES cells, as well as to study the epigenetic reversal process. Ultimate clinical application of an iPS-cell approach would largely require methods of directed differentiation of human PS cells for generating homogenous populations of lineage-specific cell types as well as eliminating risks associated with the current iPS-cell drawbacks of genetic manipulation and low efficiency/slow kinetics. Recent studies have shown that one of the previously required four genes, cMyc, is dispensable for overexpression in generating iPS cells. See, Nakagawa, M. et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts *Nature Biotechnol.* 26, 101-106 (2007); Wernig, M., Meissner, A., Cassady, J. P. & Jaenisch, R. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* 2, 10-12 (2008). However, the reprogramming efficiency was substantially reduced with also much slower reprogramming kinetics in the absence of cMyc.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method of producing an induced pluripotent stem cell from a mammalian non-pluripotent cell. In some embodiments, the method comprises contacting the cell with a TGFβ receptor/ALK5 inhibitor under conditions to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell. In some embodiments, the method of the invention further comprises contacting the cell with a glycogen synthase kinase-3 (GSK-3) inhibitor. In some embodiments, the method further comprises contacting the cell with a histone deacetylase (HDAC) inhibitor.

In some embodiments, the method comprises contacting the cell with a TGFβ receptor/ALK5 inhibitor under conditions to produce the
induced pluripotent stem cell from the mammalian non-pluripotent cell, and further comprises contacting the cell with a second agent selected from the group consisting of a MEK inhibitor and an Erk inhibitor. In some embodiments, the second agent is a MEK inhibitor. In some embodiments, the method of the invention further comprises contacting the cell with a GSK-3 inhibitor. In some embodiments, the method further comprises contacting the cell with a histone deacetylase (HDAC) inhibitor.

In some embodiments, the method further comprises contacting the cell with an agent that inhibits H3K9 methylation or promotes H3K9 demethylation, an L-type Ca channel agonist, an activator of the cAMP pathway, DNA methyltransferase (DNMT) inhibitor, a nuclear receptor ligand, a histone deacetylase (HDAC) inhibitor, an erk inhibitor, or a histone methyltransferase (HMT) inhibitor. In some embodiments, the method further comprises (a) introducing one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide into the non-pluripotent cells; and/or (b) modifying the expression of one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide into the non-pluripotent cells.

In some embodiments, the conditions without the TGFβ receptor/ALK5 inhibitor are sufficient to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell, wherein the presence of the TGFβ receptor/ALK5 inhibitor improves efficiency of the method. In some embodiments, the conditions without the TGFβ receptor/ALK5 inhibitor are not sufficient to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell.

The present invention also provides a method of producing an induced pluripotent stem cell from a mammalian non-pluripotent cell comprising contacting the cell with a GSK-3 inhibitor under conditions to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell. In some embodiments, the method further comprises contacting the cell with an agent that inhibits H3K9 methylation or promotes H3K9 demethylation.

In some embodiments, the conditions without the GSK-3 inhibitor are sufficient to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell, wherein the presence of the GSK-3 inhibitor improves efficiency of the method. In some embodiments, the conditions without the GSK-3 inhibitor are not sufficient to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell.

The present invention provides a eukaryotic cell medium comprising a TGFβ receptor/ALK5 inhibitor. In some embodiments, the medium further comprises a GSK-3 inhibitor. In some embodiments, the medium further comprises a histone deacetylase inhibitor.

In some embodiments, the medium comprises a TGFβ receptor/ALK5 inhibitor, and further comprises a second agent selected from the group consisting of a MEK inhibitor and an Erk inhibitor. In some embodiments, the second agent is a MEK inhibitor. In some embodiments, the medium further comprises a GSK-3 inhibitor. In some embodiments, the medium further comprises a histone deacetylase inhibitor.

In some embodiments, the medium further comprises an agent that inhibits H3K9 methylation or promotes H3K9 demethylation, an L-type Ca channel agonist, an activator of the cAMP pathway, DNA methyltransferase (DNMT) inhibitor, a nuclear receptor ligand, a histone deacetylase (HDAC) inhibitor, an erk inhibitor, or a histone methyltransferase (HMT) inhibitor.

In some embodiments, the medium further comprises one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide. In some embodiments, the medium does not comprise cells. In some embodiments, the medium comprises cells. In some embodiments, the cells are non-pluripotent cells. In some embodiments, at least 99% of the cells are non-pluripotent cells.

The present invention also provides a eukaryotic cell medium comprising a GSK-3 inhibitor. In some embodiments, the medium further comprises an agent that inhibits H3K9 methylation or promotes H3K9 demethylation.

The present invention further provides a kit for inducing pluripotency in non-pluripotent cells. In some embodiments, the kit comprises a TGFβ receptor/ALK5 inhibitor; and (i) one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide and/or polynucleotide encoding one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide; or (ii) a MEK inhibitor and/or an Erk inhibitor; or (iii) both (i) and (ii). In some embodiments, the kit further comprises a histone deacetylase (HDAC) inhibitor.

In some embodiments, the kit comprises a GSK-3 inhibitor; and (i) one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide and/or polynucleotide encoding one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide; or (ii) an agent that inhibits H3K9 methylation or promotes H3K9 demethylation; or (iii) both (i) and (ii).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Generation of iPS cells from defined primary neural progenitor cells by Oct4/Klf4 viral transduction and BIX01294 treatment. A comparison of the number of GFP+ iPS cell colonies generated from 3.5×10^4 primary OG2 neural progenitor cells by retroviral transduction of Oct4/Klf4/Sox2/c-Myc, Oct4/Klf4/Sox2, or Oct4/Klf4 with or without BIX01294 treatment. Abbreviations: Oct4=0; Klf4=K; Sox2=S; and c-Myc=M.

FIG. **2**. Generation of iPS cells from primary neural progenitor cells by Klf4/Sox2/c-Myc viral transduction and BIX01294 treatment. The number of GFP+ iPS cell colonies generated from 3.5×10^4 primary OG2 neural progenitor cells by retroviral transduction of Klf4/Sox2/c-Myc with or without BIX01294 treatment.

FIG. **3**. Generation of OK2B iPSCs from primary OG2 MEFs. Bar graph showing the average number of GFP^+ colony induced from OG2-MEFs in 3 independent experiments. This graph shows data for OG2 MEF cells transduced with 4 factors (Oct4, Klf4, Sox2, and cMyc; 4F); transduced with OK (OK); transduced with OK and treated with 1 μM BIX (OK+BIX); transduced with OK and treated with 1 μM BIX+2 μM BayK (OK+BIX+BayK); transduced with OK and treated with 1 μM BIX+0.04 μM RG108 (0K+BIX+RG108), n=3, error bar represents standard deviation as calculated with Excel.

FIG. **4**A-B. OK2B iPSCs have a transcriptional profile similar to the one of mESCs. (A) RT-PCR analysis of OK2B iPSCs indicated they express genes specific to pluripotent mESCs. R1 mESCs were used as positive control while OG2 MEFs were used as negative control. GAPDH was used as loading control. (B) Bisulfite sequencing revealed that OK2B iPSCs' nanog promoter is demethylated, further suggesting a reactivation of endogenous genes specific to mESCs. Schematic depiction of the cytosine present in the region of the Nanog promoter that was amplified for this analysis. Open circle indicates demethylated cytosine, while filled/black circle indicates methylated cytosine.

FIG. **5**. Treatment with BIX, BayK or a combination of both does not increase the proliferation of mES cells. Scatter graph showing R1 mES cell number after treatment with DMSO (control), 2 μM BayK, 1 μM BIX and a combination of both (BayK+BIX). n=3. The error bars represent standard

deviation as calculated in Excel. No significant difference was obtained for each treatment compared to DMSO as calculated using t-test in Excel.

FIG. **6**. RT-PCR analysis for Sox2 expression after compound treatment. $OG2^{+/-}/ROSA26^{+/-}$ (OG2) MEFs were treated with DMSO (control), 1 μM BIX, 2 μM BayK, and a combination of both for 6 days. RNA was then extracted using Qiagen RNAeasy Mini Kit. Sox2 expression was assessed through semi-quantitative PCR. OK2B iPSCs p37 and R1 were used as positive control, GAPDH as loading control.

FIG. **7**A-C. SB431542 and PD0320501 treatment enhanced iPSC generation in a dose dependent manner. (A) The 4TFs transduced fibroblast cells when treated with SB431542 at 2 μM yielded colonies with hESC-like morphology as early as 7 days after compound treatment, while untreated control or those treated with PD0320501 alone, yielded none. Y-axis represents total number of ALP^+ colonies that have characteristic hESC-like morphology; (B) The reprogramming effect of SB431542 is enhanced upon combining with 0.2 μM PD0320501 and this effect was found to be dose dependent; (C) The enhancement of SB431542 (2 μM) dependent reprogramming efficiency by PD0320501 was also found to be dose dependent. Error bars (a-c), s.d. n=4. P values by two tailed Student t-test<0.01 and 0.001 are indicated by two and three asterisks respectively.

FIG. **8**A-B. CHIR99021 promoted the reprogramming of MEFs transduced by Oct4, Sox2 and Klf4. MEFs from 129 strain were transduced with Oct4, Sox2 and Klf4 by retroviruses, and treated the next day with increasing concentrations of CHIR99021 for two week. Three weeks later, AP was detected by staining cells in monolayer (A). ROSA26+/−/OG2+/−MEFs transduced with Oct4, Sox2 and Klf4 were seeded into 6-well plates at the density of 1×10^4 cells/well (together with 10^5 cells/well CF1 feeders) and treated with CHIR99021 for two weeks. After 3 weeks of treatment, the GFP positive colonies in each well were counted (B). Error bars represent standard deviation for N=3.

FIG. **9**A-G. CHIR99021 enables the reprogramming of MEFs transduced with Oct4 and Klf4 only. ROSA26+/−/OG2+/− MEFs transduced with Oct4 and Klf4 were split into 6-well plates at the density of 10^5 cells/well and treated with 10 μM CHIR99021 for 4 weeks. (A) GFP-positive colonies before picking. (B) A total of four miPSCs-OK lines were established. (C-F) The expression of Oct4 (C), Sox2 (D), Nanog, (E) and SSEA-1 (F) by miPSCs-OK was detected by immunocytochemistry. (G) The expression of pluripotency genes by MEFs after CHIR99021 treatment was analyzed by real-time PCR. Scale bars, 20 μm. Error bars represent standard deviation for N=3.

FIG. **10**A-H. PCR analysis and in vitro differentiation of miPSCs-OK. The expression of typical endogenous pluripotency genes and transduced genes (Tg) were analyzed by RT-PCR (A). Genomic PCR revealed integration of Oct4 and Klf4 retrovirues (B). 1~4 referred to the four established miPSCs-OK lines. 100 bp DNA ladder (Invitrogen) was used as a marker. Rat iPSCs generated by Oct4/Klf4/Sox2 transductions were used as positive control (+) and MEFs were used as negative control (−). Under standard EB differentiation methods, the in vitro pluripotency of miPSCs-OK was analyzed by Immunostaining (C-E). miPSCs-OK efficiently incorporated into the ICM of a blastocyst after aggregation with an 8-cell embryo (F). Chimeric embryo (13.5 dpc) was obtained after the transfer of the aggregated embryos into a pseudo-pregnant mouse. LacZ staining showed the contribution of miPSCs-OK (G). miPSCs-OK contributed to the germline cells (GFP-positive) in male gonad tissue isolated from chimeric embryos (H). Scale bars, 20 μm.

FIG. **11**A-F. hiPSCs-OK are generated from primary human keratinocytes transduced by Oct4 and Klf4. Established hiPSC-OK clones express pluripotency markers AP (A), SSEA3 (green)/Oct4 (red; B), TRA-1-81 (green)/Nanog (red; C), and SSEA4 (green)/Sox2 (red; D). Expression of endogenous (endo) markers and viral transgenes in hiPS-OK 1, 2 and 3 was determined by real-time PCR (E). Primary human keratinocytes ("K") and Hues9 human ES cells were used as controls. Error bars represent standard deviation for N=3. The methylation status of the Oct4 promoter in primary human keratinocytes and hiPSC-OK was analyzed using bisulfite sequencing. Open circles indicate unmethylated, and filled circles indicate methylated CpG dinucleotides (F).

FIG. **12**. Real-time PCR analysis of the expression of pluripotency genes by keratinocytes after small molecule treatment. Primary human keratinocytes ("K"), keratinocytes treated with either combination of 10 μM CHIR99021 and 2 μM Parnate (2 inhibitors), or combination of 10 μM CHIR99021, 2 μM Parnate, 0.5 μM PD0325901, and 2 μM SB431542 (4 inhibitors), and Hues9 human ES cells were analyzed. Error bars represent standard deviation for N=3.

FIG. **13**A-F. hiPSCs-OK showed pluripotent potential in vitro and in vivo. Using the standard EB differentiation method, the in vitro pluripotency of hiPSCs-OK was analyzed by immunostaining (A-C). hiPSCs-OK generated full teratoma in SCID mice. Hematoxylin and eosin staining of hiPSCs-OK teratoma sections showed epithelial tube structure (endoderm), cartilage-like structure (mesoderm) and neuroepithelium-like structure (ectoderm) appear in (D-F). Scale bars, 20 μm.

FIG. **14**. A-83-01, PD0320501, and CHIR99021 treatment enhanced iPSC generation. A comparison of the number of $Oct4^+$ iPS cell colonies generated from mouse fibroblast cells treated with exogenous Oct4/Sox2/Klf4/Myc polypeptides, and with or without 0.5 μM PD0325901, 3 μM CHIR99021, and 0.5 μM A-83-01. Y-axis represents percentage of $Oct4^+$ colonies within total number of colonies.

DEFINITIONS

An "Oct polypeptide" refers to any of the naturally-occurring members of Octamer family of transcription factors, or variants thereof that maintain transcription factor activity, similar (within at least 50%, 80%, or 90% activity) compared to the closest related naturally occurring family member, or polypeptides comprising at least the DNA-binding domain of the naturally occurring family member, and can further comprise a transcriptional activation domain. Exemplary Oct polypeptides include, Oct-1, Oct-2, Oct-3/4, Oct-6, Oct-7, Oct-8, Oct-9, and Oct-11. e.g. Oct3/4 (referred to herein as "Oct4") contains the POU domain, a 150 amino acid sequence conserved among Pit-1, Oct-1, Oct-2, and uric-86. See, Ryan, A. K. & Rosenfeld, M. G. *Genes Dev.* 11, 1207-1225 (1997). In some embodiments, variants have at least 85%, 90%, or 95% amino acid sequence identity across their whole sequence compared to a naturally occurring Oct polypeptide family member such as to those listed above or such as listed in Genbank accession number NP_002692.2 (human Oct4) or NP_038661.1 (mouse Oct4). Oct polypeptides (e.g., Oct3/4) can be from human, mouse, rat, bovine, porcine, or other animals. Generally, the same species of protein will be used with the species of cells being manipulated.

A "Klf polypeptide" refers to any of the naturally-occurring members of the family of Kruppel-like factors (Klfs), zinc-finger proteins that contain amino acid sequences similar to those of the *Drosophila* embryonic pattern regulator Kruppel, or variants of the naturally-occurring members that maintain transcription factor activity similar (within at least 50%, 80%, or 90% activity) compared to the closest related naturally occurring family member, or polypeptides comprising at least the DNA-binding domain of the naturally occurring family member, and can further comprise a transcriptional activation domain. See, Dang, D. T., Pevsner, J. & Yang, V. W. *Cell Biol.* 32, 1103-1121 (2000). Exemplary Klf family members include, Klf1, Klf2, Klf3, Klf-4, Klf5, Klf6, Klf7, Klf8, Klf9, Klf10, Klf11, Klf12, Klf13, Klf14, Klf15, Klf16, and Klf17. Klf2 and Klf-4 were found to be factors capable of generating iPS cells in mice, and related genes Klf1 and Klf5 did as well, although with reduced efficiency. See, Nakagawa, et al., *Nature Biotechnology* 26:101-106 (2007). In some embodiments, variants have at least 85%, 90%, or 95% amino acid sequence identity across their whole sequence compared to a naturally occurring Klf polypeptide family member such as to those listed above or such as listed in Genbank accession number CAX16088 (mouse Klf4) or CAX14962 (human Klf4). Klf polypeptides (e.g., Klf1, Klf4, and Klf5) can be from human, mouse, rat, bovine, porcine, or other animals. Generally, the same species of protein will be used with the species of cells being manipulated. To the extent a Klf polypeptide is described herein, it can be replaced with an estrogen-related receptor beta (Essrb) polypeptide. Thus, it is intended that for each Klf polypeptide embodiment described herein, a corresponding embodiment using Essrb in the place of a Klf4 polypeptide is equally described.

A "Myc polypeptide" refers any of the naturally-occurring members of the Myc family (see, e.g., Adhikary, S. & Eilers, M. *Nat. Rev. Mol. Cell Biol.* 6:635-645 (2005)), or variants thereof that maintain transcription factor activity similar (within at least 50%, 80%, or 90% activity) compared to the closest related naturally occurring family member, or polypeptides comprising at least the DNA-binding domain of the naturally occurring family member, and can further comprise a transcriptional activation domain. Exemplary Myc polypeptides include, e.g., c-Myc, N-Myc and L-Myc. In some embodiments, variants have at least 85%, 90%, or 95% amino acid sequence identity across their whole sequence compared to a naturally occurring Myc polypeptide family member, such as to those listed above or such as listed in Genbank accession number CAA25015 (human Myc). Myc polypeptides (e.g., c-Myc) can be from human, mouse, rat, bovine, porcine, or other animals. Generally, the same species of protein will be used with the species of cells being manipulated.

A "Sox polypeptide" refers to any of the naturally-occurring members of the SRY-related HMG-box (Sox) transcription factors, characterized by the presence of the high-mobility group (HMG) domain, or variants thereof that maintain transcription factor activity similar (within at least 50%, 80%, or 90% activity) compared to the closest related naturally occurring family member, or polypeptides comprising at least the DNA-binding domain of the naturally occurring family member, and can further comprise a transcriptional activation domain. See, e.g., Dang, D. T., et al., *Int. J. Biochem. Cell Biol.* 32:1103-1121 (2000). Exemplary Sox polypeptides include, e.g., Sox1, Sox-2, Sox3, Sox4, Sox5, Sox6, Sox7, Sox8, Sox9, Sox10, Sox11, Sox12, Sox13, Sox14, Sox15, Sox17, Sox18, Sox-21, and Sox30. Sox1 has been shown to yield iPS cells with a similar efficiency as Sox2, and genes Sox3, Sox15, and Sox18 have also been shown to generate iPS cells, although with somewhat less efficiency than Sox2. See, Nakagawa, et al., *Nature Biotechnology* 26:101-106 (2007). In some embodiments, variants have at least 85%, 90%, or 95% amino acid sequence identity across their whole sequence compared to a naturally occurring Sox polypeptide family member such as to those listed above or such as listed in Genbank accession number CAA83435 (human Sox2). Sox polypeptides (e.g., Sox1, Sox2, Sox3, Sox15, or Sox18) can be from human, mouse, rat, bovine, porcine, or other animals. Generally, the same species of protein will be used with the species of cells being manipulated.

"H3K9" refers to histone H3 lysine 9. H3K9 modifications associated with gene activity include H3K9 acetylation and H3K9 modifications associated with heterochromatin, include H3K9 di-methylation or tri-methylation. See, e.g., Kubicek, et al., *Mol. Cell* 473-481 (2007).

The term "pluripotent" or "pluripotency" refers to cells with the ability to give rise to progeny that can undergo differentiation, under the appropriate conditions, into cell types that collectively demonstrate characteristics associated with cell lineages from all of the three germinal layers (endoderm, mesoderm, and ectoderm). Pluripotent stem cells can contribute to many or all tissues of a prenatal, postnatal or adult animal. A standard art-accepted test, such as the ability to form a teratoma in 8-12 week old SCID mice, can be used to establish the pluripotency of a cell population, however identification of various pluripotent stem cell characteristics can also be used to detect pluripotent cells.

"Pluripotent stem cell characteristics" refer to characteristics of a cell that distinguish pluripotent stem cells from other cells. The ability to give rise to progeny that can undergo differentiation, under the appropriate conditions, into cell types that collectively demonstrate characteristics associated with cell lineages from all of the three germinal layers (endoderm, mesoderm, and ectoderm) is a pluripotent stem cell characteristic. Expression or non-expression of certain combinations of molecular markers are also pluripotent stem cell characteristics. For example, human pluripotent stem cells express at least some, and optionally all, of the markers from the following non-limiting list: SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin, UTF-1, Oct4, Rex1, and Nanog. Cell morphologies associated with pluripotent stem cells are also pluripotent stem cell characteristics.

The term "library" is used according to its common usage in the art, to denote a collection of molecules, optionally organized and/or cataloged in such a way that individual members can be identified. Libraries can include, but are not limited to, combinatorial chemical libraries, natural products libraries, and peptide libraries.

A "recombinant" polynucleotide is a polynucleotide that is not in its native state, e.g., the polynucleotide comprises a nucleotide sequence not found in nature, or the polynucleotide is in a context other than that in which it is naturally found, e.g., separated from nucleotide sequences with which it typically is in proximity in nature, or adjacent (or contiguous with) nucleotide sequences with which it typically is not in proximity. For example, the sequence at issue can be cloned into a vector, or otherwise recombined with one or more additional nucleic acid.

"Expression cassette" refers to a polynucleotide comprising a promoter or other regulatory sequence operably linked to a sequence encoding a protein.

The terms "promoter" and "expression control sequence" are used herein to refer to an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. Promoters include constitutive and inducible promoters. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

A "heterologous sequence" or a "heterologous nucleic acid", as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous expression cassette in a cell is an expression cassette that is not endogenous to the particular host cell, for example by being linked to nucleotide sequences from an expression vector rather than chromosomal DNA, being linked to a heterologous promoter, being linked to a reporter gene, etc.

The terms "agent" or "test compound" refer to any compound useful in the screening assays described herein. An agent can be, for example, an organic compound (e.g., a small molecule such as a drug), a polypeptide (e.g., a peptide or an antibody), a nucleic acid (e.g., DNA, RNA, double-stranded, single-stranded, an oligonucleotide, antisense RNA, small inhibitory RNA, micro RNA, a ribozyme, etc.), an oligosaccharide, a lipid. Usually, the agents used in the present screening methods have a molecular weight of less than 10,000 daltons, for example, less than 8000, 6000, 4000, 2000 daltons, e.g., between 50-1500, 500-1500, 200-2000, 500-5000 daltons. The test compound can be in the form of a library of test compounds, such as a combinatorial or randomized library that provides a sufficient range of diversity. Test compounds are optionally linked to a fusion partner, e.g., targeting compounds, rescue compounds, dimerization compounds, stabilizing compounds, addressable compounds, and other functional moieties. Conventionally, new chemical entities with useful properties are generated by identifying a test compound (called a "lead compound") with some desirable property or activity, e.g., ability to induce pluripotency under certain conditions such as are described herein, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Often, high throughput screening (HTS) methods are employed for such an analysis.

The terms "nucleic acid" and "polynucleotide" are used interchangeably herein to refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)).

"Inhibitors," "activators," and "modulators" of expression or of activity are used to refer to inhibitory, activating, or modulating molecules, respectively, identified using in vitro and in vivo assays for expression or activity of a described target protein (or encoding polynucleotide), e.g., ligands, agonists, antagonists, and their homologs and mimetics. The term "modulator" includes inhibitors and activators. Inhibitors are agents that, e.g., inhibit expression or bind to, partially or totally block stimulation or protease inhibitor activity, decrease, prevent, delay activation, inactivate, desensitize, or down regulate the activity of the described target protein, e.g., antagonists. Activators are agents that, e.g., induce or activate the expression of a described target protein or bind to, stimulate, increase, open, activate, facilitate, enhance activation or protease inhibitor activity, sensitize or up regulate the activity of described target protein (or encoding polynucleotide), e.g., agonists. Modulators include naturally occurring and synthetic ligands, antagonists and agonists (e.g., small chemical molecules, antibodies and the like that function as either agonists or antagonists). Such assays for inhibitors and activators include, e.g., applying putative modulator compounds to cells expressing the described target protein and then determining the functional effects on the described target protein activity, as described above. Samples or assays comprising described target protein that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of effect. Control samples (untreated with modulators) are assigned a relative activity value of 100%. Inhibition of a described target protein is achieved when the activity value relative to the control is about 80%, optionally 50% or 25, 10%, 5% or 1%. Activation of the described target protein is achieved when the activity value relative to the control is 110%, optionally 150%, optionally 200, 300%, 400%, 500%, or 1000-3000% or more higher.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

The present invention is based, in part, on the discovery that small molecules such as a TGFβ receptor/ALK5 inhibitor, a MEK inhibitor or an Erk inhibitor, a GSK-3 inhibitor, or a combination thereof, are effective in inducing pluripotent stem cells (iPS). Small molecules can be included to "complement" or replace what is generally otherwise understood as a necessary expression of one of these proteins to result in pluripotent cells; and/or to improve the efficiency of a process for generating pluripotent cells (e.g., iPS cells).

As shown in a large number of examples herein, the inclusion of ALK5 or GSK-3 inhibitors alone, or in combination with each other or ALK5 inhibitor/GSK-3 inhibitor/

Mek inhibitor combinations have all been shown to greatly improve efficiency of traditional four transcription factor iPSC generation, either as introduced by nucleic acid vectors or when reprogrammed in the presence of exogenous transcription factor proteins.

Moreover, the Examples provide numerous instances in which fewer than four (and in some embodiments, only one) transcription factor is introduced into cells that are subsequently reprogrammed into pluripotent cells. The details of such "replacement" of transcription factors is detailed more below, where it is shown that reprogramming can be successfully achieved with exclusion of a SOX polypeptide by including a GSK-3 inhibitor, with exclusion of a Myc polypeptide by inclusion of an ALK5 inhibitor, with exclusion of a Sox and Myc polypeptide by inclusion of an H3K9 histone methylation inhibitor and a Mek inhibitor, and with exclusion of an Oct polypeptide by inclusion of an H3K9 histone methylation inhibitor.

The present invention also provides induced pluripotent cells in which at least some of the iPS transcription factors are expressed at endogenous or lower levels and nevertheless are pluripotent. For example, in some embodiments cells that endogenously express Sox2 can be induced to pluripotency by introduction and heterologous expression of only Oct4 and Klf4.

II. Induction of Pluripotent Stem Cells

A. Small Molecules and Combinations Thereof for Inducing Pluripotency and Improving Reprogramming Efficiency

As discussed above, small molecules can be included to "complement" or replace what is generally otherwise understood as a necessary expression of pluripotency proteins to result in pluripotent cells; and/or to improve the efficiency of a process for generating pluripotent cells (e.g., iPS cells). By contacting a cell with an agent that functionally replaces one of the transcription factors, it is possible to generate pluripotent cells with all of the above-listed transcription factors except for the transcription factor replaced or complemented by the agent. Therefore, in some embodiments, some but not all of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide (e.g., combinations of one, two or three of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide) are introduced into the non-pluripotent cells. In some embodiments, all four polypeptides are introduced into the non-pluripotent cells. For examples, the non-pluripotent cells can be retrovirally transduced with Oct4, Klf4, Sox2 and c-Myc; or Oct4, Klf4, and Sox2; or Oct4 and Klf4; or Oct4 alone. The transcription factors can be introduced by contacting the transcription factors with the non-pluripotent cells, e.g., by using an exogenous transcription factor comprising an amino acid sequence that enhances transport across cell membranes.

Further, in some embodiments, small molecules can improve the efficiency of a process for generating pluripotent cells (e.g., iPS cells). For example, improved efficiency can be manifested by speeding the time to generate such pluripotent cells (e.g., by shortening the time to development of pluripotent cells by at least a day compared to a similar or same process without the small molecule). Alternatively, or in combination, a small molecule can increase the number of pluripotent cells generated by a particular process (e.g., increasing the number in a given time period by at least 10%, 30%, 50%, 100%, 200%, 500%, etc. compared to a similar or same process without the small molecule). In some embodiments, a 2-fold to 20-fold improvement in reprogramming efficiency is observed. In some embodiments, reprogramming efficiency is improved by more than 20 fold. In some embodiments, a more than 100 fold improvement in efficiency is observed over the conventional method (e.g., a more than 100 fold increase in the number of pluripotent cells generated).

We found that TGFβ receptor/ALK5 inhibitors, alone or in combination with other small molecules, are effective in inducing pluripotency in non-pluripotent mammalian cells. Accordingly, the present invention provides for a method of producing a pluripotent stem cell from a mammalian non-pluripotent cell by contacting the cell with a TGFβ receptor/ALK5 inhibitor under conditions to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell. For example, some conditions are sufficient to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell with or without the TGFβ receptor/ALK5 inhibitor. The addition of the TGFβ receptor/ALK5 inhibitor improves reprogramming efficiency. Some conditions, without the TGFβ receptor/ALK5 inhibitor, are not sufficient to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell.

We also found that (1) the combination of a TGFβ receptor/ALK5 inhibitor and a MEK inhibitor or an Erk inhibitor; or (2) the combination of a TGFβ receptor/ALK5 inhibitor and a GSK-3 inhibitor; or (3) the combination of a TGFβ receptor/ALK5 inhibitor, a MEK inhibitor or an Erk inhibitor, and a GSK-3 inhibitor; is effective in inducing pluripotency in non-pluripotent mammalian cells. Accordingly, the present invention provides for a method of producing a pluripotent stem cell from a mammalian non-pluripotent cell by contacting the cell with any of the above-described combinations under conditions to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell.

We also found that GSK-3 inhibitors, alone or in combination with other small molecules, are effective in inducing pluripotency in non-pluripotent mammalian cells. Accordingly, the present invention provides for a method of producing a pluripotent stem cell from a mammalian non-pluripotent cell by contacting the cell with a GSK-3 inhibitor under conditions to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell. The GSK-3 inhibitor, e.g., CHIR99021, can be effective in inducing pluripotency by either replacing one of the transcription factor or by improving reprogramming efficiency, or via both mechanisms. For example, some conditions are sufficient to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell with or without the GSK-3 inhibitor. The addition of the GSK-3 inhibitor improves reprogramming efficiency. Some conditions, without the GSK-3 inhibitor, are not sufficient to produce the induced pluripotent stem cell from the mammalian non-pluripotent cell.

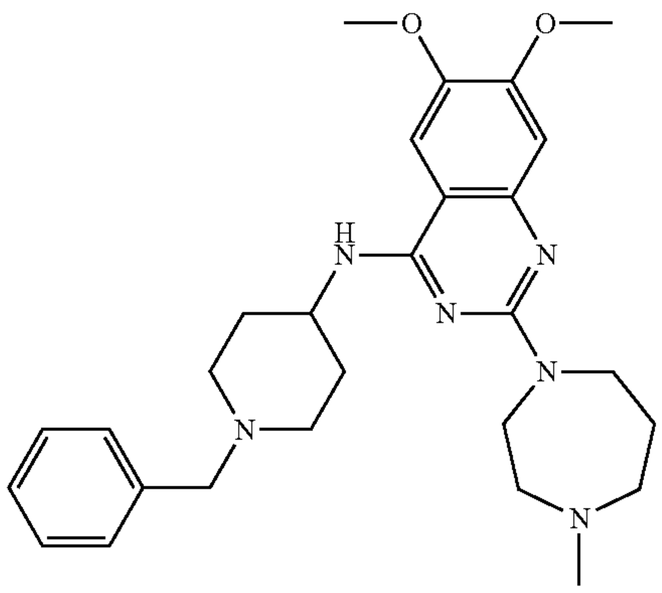
We further discovered that some compounds are effective in inducing pluripotency in non-pluripotent mammalian cells when used together with either (1) a TGFβ receptor/ALK5 inhibitor alone or in combination with other small molecules; or (2) a GSK-3 inhibitor alone or in combination with other small molecules. Exemplary compounds include an agent that inhibits H3K9 methylation or promotes H3K9 demethylation, an L-type Ca channel agonist, an activator of the cAMP pathway, a DNA methyltransferase (DNMT) inhibitor, a nuclear receptor ligand, a histone deacetylase (HDAC) inhibitor, an erk inhibitor, or a histone methyltransferase (HMT) inhibitor. Again, these compounds can be effective in inducing pluripotency by either replacing one of the transcription factor or by improving reprogramming

efficiency, or via both mechanisms. In some embodiments, the combination of a TGFβ receptor/ALK5 inhibitor and a histone deacetylase (HDAC) inhibitor (e.g., sodium butyrate or valproic acid) is effective in inducing pluripotency. In some embodiments, the combination effective in inducing pluripotency comprises a TGFβ receptor/ALK5 inhibitor, a MEK inhibitor or an Erk inhibitor, and a histone deacetylase (HDAC) inhibitor.

In some embodiments, when inducing pluripotency using either (1) a TGFβ receptor/ALK5 inhibitor alone or in combination with other small molecules; or (2) a GSK-3 inhibitor alone or in combination with other small molecules, one or more of a transcription factor is introduced into the non-pluripotent cells. Alternatively, expression of one or more of a transcription factor is modified. Transcription factors include, for example, an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide.

The cell into which a protein of interest is introduced can be a mammalian cell. The cells can be human or non-human (e.g., primate, rat, mouse, rabbit, bovine, dog, cat, pig, etc.). The cell can be, e.g., in culture or in a tissue, fluid, etc. and/or from or in an organism. Cells that can be induced to pluripotency include, but are not limited to, keratinocyte cells, hair follicle cells, HUVEC (Human Umbilical Vein Endothelial Cells), cord blood cells, neural progenitor cells and fibroblasts.

Agents that inhibit H3K9 methylation include agents that inhibit methylases (also known as methyl transferases) that target H3K9. For example, G9a histone methyltransferase methylates H3K9 and inhibition of G9a histone methyltransferase is known to reduce methylation of H3K9. See, e.g., Kubicek, et al., *Mol. Cell* 473-481 (2007). An example of a G9a histone methyltransferase useful according to the methods of the invention is BIX01294 (see, e.g., Kubicek, et al., *Mol. Cell* 473-481 (2007)), or salts, hydrates, isoforms, racemates, solvates and prodrug forms thereof. Bix01294 is displayed below:



BIX-01294

The Bix01294 compounds of the present invention also include the salts, hydrates, solvates and prodrug forms. Bix01294 possesses asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are all intended to be encompassed within the scope of the present invention. For example, the compound of the present invention can be the R-isomer or the S-isomer, or a mixture thereof. In addition, the compound of the present invention can be the E-isomer or the Z-isomer, or a combination thereof.

In some embodiments, the agent that inhibits H3K9 methylation is a substrate analog of a histone methyl transferase. The substrate of a number of methyl transferases is S-adenosyl-methionine (SAM). Thus, in some embodiments, the agent that inhibits H3K9 methylation is a SAM analog. Exemplary SAM analogs include, but are not limited to, methylthio-adenosine (MTA), sinefungin, and S-adenosyl-homocysteine (SAH). In other embodiments, the agent that inhibits H3K9 methylation does not compete with SAM on a histone methyl transferase.

The resulting pluripotent cells (from heterologous expression and/or small molecule "replacement") can develop into many or all of the three major tissue types: endoderm (e.g., interior gut lining), mesoderm (e.g., muscle, bone, blood), and ectoderm (e.g., epidermal tissues and nervous system), but, optionally, may show restrictions to their developmental potential (e.g., they may not form placental tissue, or other cell types of a defined lineage). The cells can be human or non-human (e.g., primate, rat, mouse, rabbit, bovine, dog, cat, pig, etc.).

In other embodiments, BIX01294, or other agents that inhibit H3K9 methylation or promote H3K9 demethylation can be used to induce pluripotency in cells that were previously not pluripotent. In some embodiments, an agent that inhibits H3K9 methylation is used to induce Oct4 expression in cells, or at least alterations in Oct4 promoter DNA methylation and/or histone methylation to allow for induction of cells into pluripotency. Thus, in some embodiments, cells that are not initially pluripotent cells are contacted with an agent that inhibits H3K9 methylation to induce the cells to become pluripotent. Indeed, without intending to limit the scope of the invention to a particular mode of action, the inventors believe that contacting non-pluripotent cells with an agent that inhibits H3K9 methylation or promotes H3K9 demethylation will improve any method of inducing cells to pluripotency. For example, the agent that inhibits H3K9 methylation can be contacted to a non-pluripotent cell and induced to pluripotency in a method comprising contacting the non-pluripotent cells with an agent that inhibits H3K9 methylation, optionally also contacting the cells with one or more of an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor, wherein each compound is included in an amount sufficient in improve the efficiency of induction. In some embodiments as described in this paragraph, Oct4 only, or Oct4/Klf4, or Sox2/Klf4 are further heterologously expressed in the non-pluripotent cells resulting in induction of pluripotency following contacting with agents as described herein.

In some embodiments, a non-pluripotent cell is induced to pluripotency in a method comprising contacting the non-pluripotent cells with a GSK3 inhibitor, optionally also contacting the cells with one or more of an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor, wherein each compound is included in an amount sufficient in improve the efficiency of induction. In other embodiments, a non-pluripotent cell is induced to pluripotency in a method comprising contacting the non-pluripotent cells with a TGFβ receptor/ALK5 inhibitor, optionally also contacting the cells with one or more of an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibi-

tor; a MEK inhibitor, a HDAC inhibitor; or an Erk inhibitor, wherein each compound is included in an amount sufficient in improve the efficiency of induction. In other embodiments, a non-pluripotent cell is induced to pluripotency in a method comprising contacting the non-pluripotent cells with a HDAC inhibitor, optionally also contacting the cells with one or more of an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, or an Erk inhibitor, wherein each compound is included in an amount sufficient in improve the efficiency of induction. In other embodiments, a non-pluripotent cell is induced to pluripotency in a method comprising contacting the non-pluripotent cells with a MEK inhibitor, optionally also contacting the cells with one or more of an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor, wherein each compound is included in an amount sufficient in improve the efficiency of induction. In other embodiments, a non-pluripotent cell is induced to pluripotency in a method comprising contacting the non-pluripotent cells with two, three, four, five, six, seven, eight, nine, or each of a MEK inhibitor, an L-type Ca channel agonist; an agent that inhibits H3K9 methylation, an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor, wherein each compound is included in an amount sufficient in improve the efficiency of induction. In some embodiments as described in this paragraph, Oct4 only, or Oct4/Klf4, or Sox2/Klf4 are further heterologously expressed in the non-pluripotent cells resulting in induction of pluripotency following contacting with agents as described herein.

Exemplary L-type calcium channel agonists include, but are not limited to, BayK8644 (see, e.g., Schramm, et al., *Nature* 303:535-537 (1983)), Dehydrodidemnin B (see, e.g., U.S. Pat. No. 6,030,943), FPL 64176 (FPL) (see, e.g., Liwang, et al., *Neuropharmacology* 45:281-292 (2003)), S(+)-PN 202-791 (see, e.g., Kennedy, et al., *Neuroscience* 49:937-44 (1992)) and CGP 48506 (see, e.g., Chahine, et al., *Canadian Journal of Physiology and Pharmacology* 81:135-141 (2003)).

Exemplary activators of the cAMP pathway include, but are not limited to, forskolin (see, e.g., Liang, et al., *Endocrinology* 146: 4437-4444 (2005)), FSH (see, Liang, supra), milrinone (see, Liang, supra), cilostamide (see, Liang, supra), rolipram (see, Liang, supra), dbcAMP (see, Liang, supra) and 8-Br-cAMP (see, Liang, supra).

Exemplary DNA methyltransferase (DNMT) inhibitors can include antibodies that bind, dominant negative variants of, and siRNA and antisense nucleic acids that suppress expression of DNMT. DNMT inhibitors include, but are not limited to, RG108 (available, e.g., from Sigma-Aldrich), 5-aza-C (5-azacitidine or azacitidine) (see, e.g., Schermelleh, et al., *Nature Methods* 2:751-6 (2005)), 5-aza-2'-deoxycytidine (5-aza-CdR) (see, e.g., Zhu, *Clinical Medicinal Chemistry* 3(3):187-199 (2003)), decitabine (see, e.g., Gore, *Nature Clinical Practice Oncology* 2:S30-S35 (2005)), doxorubicin (see, e.g., Levenson, *Molecular Pharmacology* 71:635-637 (2007)), EGCG ((−)-epigallocatechin-3-gallate) (see, e.g., Fang, et al., *Cancer Research* 63:7563-7570 (2003)), RG108 (see, e.g., Carninci, et al., WO2008/126932, incorporated herein by reference) and zebularine (see, Carninci, supra).

Exemplary nuclear receptor ligands, i.e., agonists, antagonists, activators and/or repressors of nuclear receptors, can modulate local gene expression or transcription at the site of delivery. Nuclear receptor agonist (and also nuclear receptor antagonists) can be used. In some embodiments, nuclear receptors are co-regulators of transcription. Activation or inhibition of certain nuclear receptors regulate epigenetic states of specific gene loci where they bind. The inventors have found that dexamethasone (e.g., at 1 μM, a glucocorticoid receptor agonist), ciglitazone and Fmoc-Leu (both used at 5 μM) (a PPAR agonist), and Bexarotene (e.g., at (3 μM) (a RXR antagonist) can enhance cellular reprogramming. Representative nuclear receptor ligands include, but are not limited to, estradiol (e.g., 17-beta estradiol), all-trans retinoic acid, 13-cis retinoic acid, dexamethasone, clobetasol, androgens, thyroxine, vitamin D3 glitazones, troglitazone, pioglitazone, rosiglitazone, prostaglandins, and fibrates (e.g., bezafibrate, ciprofibrate, gemfibrozil, fenofibrate and clofibrate). Furthermore, the activity of endogenous ligands (such as the hormones estradiol and testosterone) when bound to their cognate nuclear receptors is normally to upregulate gene expression. This upregulation or stimulation of gene expression by the ligand can be referred to as an agonist response. The agonistic effects of endogenous hormones can also be mimicked by certain synthetic ligands, for example, the glucocortocoid receptor anti-inflammatory drug dexamethasone. Agonist ligands function by inducing a conformation of the receptor which favors coactivator binding. (See, e.g., WO08011093A incorporate herein by reference.)

Inhibitors of GSK3 (sometimes referred herein as "GSK") can include antibodies that bind, dominant negative variants of, and siRNA and antisense nucleic acids that target GSK3. Specific examples of GSK3 inhibitors include, but are not limited to, Kenpaullone, 1-Azakenpaullone, CHIR99021, CHIR98014, AR-A014418 (see, e.g., Gould, et al., *The International Journal of Neuropsychopharmacology* 7:387-390 (2004)), CT 99021 (see, e.g., Wagman, *Current Pharmaceutical Design* 10:1105-1137 (2004)), CT 20026 (see, Wagman, supra), SB216763 (see, e.g., Martin, et al., *Nature Immunology* 6:777-784 (2005)), AR-A014418 (see, e.g., Noble, et al., *PNAS* 102:6990-6995 (2005)), lithium (see, e.g., Gould, et al., *Pharmacological Research* 48: 49-53 (2003)), SB 415286 (see, e.g., Frame, et al., *Biochemical Journal* 359:1-16 (2001)) and TDZD-8 (see, e.g., Chin, et al., *Molecular Brain Research,* 137 (1-2):193-201 (2005)). Further exemplary GSK3 inhibitors available from Calbiochem (see, e.g., Dalton, et al., WO2008/094597, herein incorporated by reference), include but are not limited to BIO (2'Z,3'£)-6-Bromoindirubin-3'-oxime (GSK3 Inhibitor IX); BIO-Acetoxime (2'Z,3'E)-6-Bromoindirubin-3'-acetoxime (GSK3 Inhibitor X); (5-Methyl-1H-pyrazol-3-yl)-(2-phenylquinazolin-4-yl)amine (GSK3-Inhibitor XIII); Pyridocarbazole-cyclopenadienylruthenium complex (GSK3 Inhibitor XV); TDZD-8 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (GSK3beta Inhibitor I); 2-Thio(3-iodobenzyl)-5-(1-pyridyl)[1,3,4]-oxadiazole (GSK3beta Inhibitor II); OTDZT 2,4-Dibenzyl-5-oxothiadiazolidine-3-thione (GSK3beta Inhibitor III); alpha-4-Dibromoacetophenone (GSK3beta Inhibitor VII); AR-AO 14418 N-(4-Methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl)urea (GSK-3beta Inhibitor VIII); 3-(1-(3-Hydroxypropyl)-1H-pyrrolo[2,3-b] pyridin-3-yl]-4-pyrazin-2-yl-pyrrole-2,5-dione (GSK-3beta Inhibitor XI); TWSl 19 pyrrolopyrimidine compound (GSK3beta Inhibitor XII); L803 H-KEAPPAPPQSpP-NH2 (SEQ ID NO:1) or its Myristoylated form (GSK3beta Inhibitor XIII); 2-Chloro-1-(4,5-dibromo-thiophen-2-yl)-ethanone

(GSK3beta Inhibitor VI); AR-A0144-18; SB216763; and SB415286. Residues of GSK3b that interact with inhibitors have been identified. See, e.g., Bertrand et al., *J. Mol Biol.* 333(2): 393-407 (2003). GSK3 inhibitors can activate, for example, the Wnt/β-catenin pathway. Many of β-catenin downstream genes co-regulate pluripotency gene networks. For example, a GSK inhibitor activates cMyc expression as well as enhances its protein stability and transcriptional activity. Thus, in some embodiments, GSK3 inhibitors can be used to stimulate endogenous Myc polypeptide expression in a cell, thereby eliminating the need for Myc expression to induce pluripotency.

Inhibitors of MEK can include antibodies to, dominant negative variants of, and siRNA and antisense nucleic acids that suppress expression of, MEK. Specific examples of MEK inhibitors include, but are not limited to, PD0325901, (see, e.g., Rinehart, et al., *Journal of Clinical Oncology* 22: 4456-4462 (2004)), PD98059 (available, e.g., from Cell Signaling Technology), U0126 (available, for example, from Cell Signaling Technology), SL 327 (available, e.g., from Sigma-Aldrich), ARRY-162 (available, e.g., from Array Biopharma), PD184161 (see, e.g., Klein, et al., *Neoplasia* 8:1-8 (2006)), PD184352 (CI-1040) (see, e.g., Mattingly, et al., *The Journal of Pharmacology and Experimental Therapeutics* 316:456-465 (2006)), sunitinib (see, e.g., Voss, et al., US2008004287 incorporated herein by reference), sorafenib (see, Voss supra), Vandetanib (see, Voss supra), pazopanib (see, e.g., Voss supra), Axitinib (see, Voss supra) and PTK787 (see, Voss supra).

Currently, several MEK inhibitors are undergoing clinical trial evaluations. CI-1040 has been evaluate in Phase I and II clinical trials for cancer (see, e.g., Rinehart, et al., *Journal of Clinical Oncology* 22(22):4456-4462 (2004)). Other MEK inhibitors being evaluated in clinical trials include PD184352 (see, e.g., English, et al., *Trends in Pharmaceutical Sciences* 23(1):40-45 (2002)), BAY 43-9006 (see, e.g., Chow, et al., *Cytometry* (*Communications in Clinical Cytometry*) 46:72-78 (2001)), PD-325901 (also PD0325901), GSK1120212, ARRY-438162, RDEA119, AZD6244 (also ARRY-142886 or ARRY-886), RO5126766, XL518 and AZD8330 (also ARRY-704). (See, e.g., information from the National Institutes of Health located on the World Wide Web at clinicaltrials.gov as well as information from the Nation Cancer Institute located on the World Wide Web at cancer.gov/clinicaltrials.

TGFβ receptor (e.g., ALK5) inhibitors can include antibodies to, dominant negative variants of, and antisense nucleic acids that suppress expression of, TGFβ receptors (e.g., ALK5). Exemplary TGFβ receptor/ALK5 inhibitors include, but are not limited to, SB431542 (see, e.g., Inman, et al., *Molecular Pharmacology* 62(1):65-74 (2002)), A-83-01, also known as 3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide (see, e.g., Tojo, et al., *Cancer Science* 96(11):791-800 (2005), and commercially available from, e.g., Toicris Bioscience); 2-(3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine, Wnt3a/BIO (see, e.g., Dalton, et al., WO2008/094597, herein incorporated by reference), BMP4 (see, Dalton, supra), GW788388 (-{4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl] pyridin-2-yl}-N-(tetrahydro-2H-pyran-4-yl)benzamide) (see, e.g., Gellibert, et al., *Journal of Medicinal Chemistry* 49(7):2210-2221 (2006)), SM16 (see, e.g., Suzuki, et al., *Cancer Research* 67(5):2351-2359 (2007)), IN-1130 (3-((5-(6-methylpyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imidazol-2-yl)methyl)benzamide) (see, e.g., Kim, et al., *Xenobiotica* 38(3):325-339 (2008)), GW6604 (2-phenyl-4-(3-pyridin-2-yl-1H-pyrazol-4-yl)pyridine) (see, e.g., de Gouville, et al., *Drug News Perspective* 19(2):85-90 (2006)), SB-505124 (2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride) (see, e.g., DaCosta, et al., *Molecular Pharmacology* 65(3):744-752 (2004)) and pyrimidine derivatives (see, e.g., those listed in Stiefl, et al., WO2008/006583, herein incorporated by reference). Further, while "an ALK5 inhibitor" is not intended to encompass non-specific kinase inhibitors, an "ALK5 inhibitor" should be understood to encompass inhibitors that inhibit ALK4 and/or ALK7 in addition to ALK5, such as, for example, SB-431542 (see, e.g., Inman, et al., *J, Mol. Phamacol.* 62(1): 65-74 (2002). Without intending to limit the scope of the invention, it is believed that ALK5 inhibitors affect the mesenchymal to epithelial conversion/transition (MET) process. TGFβ/activin pathway is a driver for epithelial to mesenchymal transition (EMT). Therefore, inhibiting the TGFβ/activin pathway can facilitate MET (i.e. reprogramming) process.

In view of the data herein showing the effect of inhibiting ALK5, it is believed that inhibition of the TGFβ/activin pathway will have similar effects. Thus, any inhibitor (e.g., upstream or downstream) of the TGFβ/activin pathway can be used in combination with, or instead of, ALK5 inhibitors as described in each paragraph herein. Exemplary TGFβ/activin pathway inhibitors include but are not limited to: TGFβ receptor inhibitors, inhibitors of SMAD 2/3 phosphorylation, inhibitors of the interaction of SMAD 2/3 and SMAD 4, and activators/agonists of SMAD 6 and SMAD 7. Furthermore, the categorizations described below are merely for organizational purposes and one of skill in the art would know that compounds can affect one or more points within a pathway, and thus compounds may function in more than one of the defined categories.

TGFβ receptor inhibitors can include antibodies to, dominant negative variants of and siRNA or antisense nucleic acids that target TGFβ receptors. Specific examples of inhibitors include but are not limited to SU5416; 2-(5-benzo [1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methyl-pyridine hydrochloride (SB-505124); lerdelimumb (CAT-152); metelimumab (CAT-192); GC-1008; ID11; AP-12009; AP-11014; LY550410; LY580276; LY364947; LY2109761; SB-505124; SB-431542; SD-208; SM16; NPC-30345; Ki26894; SB-203580; SD-093; Gleevec; 3,5,7,2',4'-pentahydroxyflavone (Morin); activin-M108A; P144; soluble TBR2-Fc; and antisense transfected tumor cells that target TGFβ receptors. (See, e.g., Wrzesinski, et al., *Clinical Cancer Research* 13(18):5262-5270 (2007); Kaminska, et al., *Acta Biochimica Polonica* 52(2):329-337 (2005); and Chang, et al., *Frontiers in Bioscience* 12:4393-4401 (2007).)

Inhibitors of SMAD 2/3 phosphorylation can include antibodies to, dominant negative variants of and antisense nucleic acids that target SMAD2 or SMAD3. Specific examples of inhibitors include PD169316; SB203580; SB-431542; LY364947; A77-01; and 3,5,7,2',4'-pentahydroxyflavone (Morin). (See, e.g., Wrzesinski, supra; Kaminska, supra; Shimanuki, et al., *Oncogene* 26:3311-3320 (2007); and Kataoka, et al., EP1992360, incorporated herein by reference.)

Inhibitors of the interaction of SMAD 2/3 and smad4 can include antibodies to, dominant negative variants of and antisense nucleic acids that target SMAD2, SMAD3 and/or smad4. Specific examples of inhibitors of the interaction of SMAD 2/3 and SMAD4 include but are not limited to Trx-SARA, Trx-xFoxH1b and Trx-Lef1. (See, e.g., Cui, et al., *Oncogene* 24:3864-3874 (2005) and Zhao, et al., *Molecular Biology of the Cell,* 17:3819-3831 (2006).)

Activators/agonists of SMAD 6 and SMAD 7 include but are not limited to antibodies to, dominant negative variants of and antisense nucleic acids that target SMAD 6 or SMAD 7. Specific examples of inhibitors include but are not limited to smad7-as PTO-oligonucleotides. (See, e.g., Miyazono, et al., U.S. Pat. No. 6,534,476, and Steinbrecher, et al., US2005119203, both incorporated herein by reference.)

Exemplary HDAC inhibitors can include antibodies that bind, dominant negative variants of, and siRNA and antisense nucleic acids that target HDAC. HDAC inhibitors include, but are not limited to, TSA (trichostatin A) (see, e.g., Adcock, *British Journal of Pharmacology* 150:829-831 (2007)), VPA (valproic acid) (see, e.g., Munster, et al., *Journal of Clinical Oncology* 25:18S (2007): 1065), sodium butyrate (NaBu) (see, e.g., Han, et al., *Immunology Letters* 108:143-150 (2007)), SAHA (suberoylanilide hydroxamic acid or vorinostat) (see, e.g., Kelly, et al., *Nature Clinical Practice Oncology* 2:150-157 (2005)), sodium phenylbutyrate (see, e.g., Gore, et al., *Cancer Research* 66:6361-6369 (2006)), depsipeptide (FR901228, FK228) (see, e.g., Zhu, et al., *Current Medicinal Chemistry* 3(3):187-199 (2003)), trapoxin (TPX) (see, e.g., Furumai, et al., *PNAS* 98(1):87-92 (2001)), cyclic hydroxamic acid-containing peptide 1 (CHAP1) (see, Furumai supra), MS-275 (see, e.g., Carninci, et al., WO2008/126932, incorporated herein by reference)), LBH589 (see, e.g., Goh, et al., WO2008/108741 incorporated herein by reference) and PXD101 (see, Goh, supra). In general at the global level, pluripotent cells have more histone acetylation, and differentiated cells have less histone acetylation. Histone acetylation is also involved in histone and DNA methylation regulation. In some embodiments, HDAC inhibitors facilitate activation of silenced pluripotency genes.

Exemplary ERK inhibitors include PD98059 (see, e.g., Zhu, et al., *Oncogene* 23:4984-4992 (2004)), U0126 (see, Zhu, supra), FR180204 (see, e.g., Ohori, Drug News Perspective 21(5):245-250 (2008)), sunitinib (see, e.g., Ma, et al., US2008004287 incorporated herein by reference), sorafenib (see, Ma, supra), Vandetanib (see, Ma, supra), pazopanib (see, Ma, supra), Axitinib (see, Ma, supra) and PTK787 (see, Ma, supra).

Once pluripotency proteins have been introduced into the cells and/or the cells have been contacted with the one or more agents, the cells can be optionally screened for characteristics of pluripotent stem cells, thereby identifying those cells in a mixture that are pluripotent. Such cells can be, for example, isolated from the other cells and used further as appropriate.

B. Heterologous/Endogenous Expression

To date, a large number of different methods and protocols have been established for inducing non-pluripotent mammalian cells into induced pluripotent stem cells (iPSCs). iPSCs are similar to ESCs in morphology, proliferation, and pluripotency, judged by teratoma formation and chimaera contribution. It is believed that the agents described herein (including but not limited to an ALK5 inhibitor, a GSK-3 inhibitor, a Mek inhibitor, and combinations thereof, optionally in combination with an epigenetic modifier (e.g., an agent that inhibits H3K9 histone methylation or an HDAC inhibitor, for example)), will improve essentially any reprogramming protocol for generating iPSCs. Reprogramming protocols that can be improved are believed to include those involving introduction of one or more reprogramming transcription factors selected from an Oct polypeptide (including but not limited to Oct 3/4), a Sox polypeptide (including but not limited to Sox2), a Klf polypeptide (including but not limited to Klf4) and/or a My c polypeptide (including but not limited to c-Myc). The reprogramming factors can be introduced into the cells, for example, by expression from a recombinant expression cassette that has been introduced into the target cell, or by incubating the cells in the presence of exogenous reprogramming transcription factor polypeptides such that the polypeptides enter the cell.

Studies have shown that retroviral transduction of mouse fibroblasts with four transcription factors that are highly expressed in ESCs (Oct-3/4, Sox2, KLF4 and c-Myc) generate induced pluripotent stem (iPS) cells. See, Takahashi, K. & Yamanaka, S. *Cell* 126, 663-676 (2006); Okita, K., Ichisaka, T. & Yamanaka, S. *Nature* 448, 313-317 (2007); Wernig, M. et al. *Nature* 448, 318-324 (2007); Maherali, N. et al. *Cell Stem Cell* 1, 55-70 (2007); Meissner, A., Wernig, M. & Jaenisch, R. *Nature Biotechnol.* 25, 1177-1181 (2007); Takahashi, K. et al. *Cell* 131, 861-872 (2007); Yu, J. et al. *Science* 318, 1917-1920 (2007); Nakagawa, M. et al. *Nature Biotechnol.* 26, 101-106 (2007); Wernig, M., Meissner, A., Cassady, J. P. & Jaenisch, R. *Cell Stem Cell.* 2, 10-12 (2008). Such methods are believed to be improved with the inclusion of agents as described herein.

To address the safety issues that arise from target cell genomes harboring integrated exogenous sequences, a number of modified genetic protocols have been further developed and can be used according to the present invention. These protocols produce iPS cells with potentially reduced risks, and include non-integrating adenoviruses to deliver reprogramming genes (Stadtfeld, M., et al. (2008) *Science* 322, 945-949), transient transfection of reprogramming plasmids (Okita, K., et al. (2008) *Science* 322, 949-953), piggyBac transposition systems (Woltjen, K., et al. (2009). *Nature* 458, 766-770, Yusa et al. (2009) *Nat. Methods* 6:363-369, Kaji, K., et al. (2009) *Nature* 458, 771-775), Cre-excisable viruses (Soldner, F., et al. (2009) *Cell* 136, 964-977), and oriP/EBNA1-based episomal expression system (Yu, J., et al. (2009) *Science* DOI: 10.1126). In some embodiments, agents as described herein are incubated with cells in any of the protocols described above.

As noted above, reprogramming can involve target cells being cultured in the presence of one or more proteins under conditions to allow for introduction of the proteins into the cell. See, e.g., Zhou H et al., *Cell Stem Cell.* 2009 May 8; 4(5):381-4; WO/2009/117439. One can introduce an exogenous polypeptide (i.e., a protein provided from outside the cell and/or that is not produced by the cell) into the cell by a number of different methods that do not involve introduction of a polynucleotide encoding the polypeptide. In some embodiments, the exogenous proteins comprise the transcription factor polypeptide of interest linked (e.g., linked as a fusion protein or otherwise covalently or non-covalently linked) to a polypeptide that enhances the ability of the transcription factor to enter the cell (and in some embodiments the cell nucleus).

In some embodiments of the invention, non-pluripotent cells are identified that endogenously express at least one (and optionally, two or three of) proteins from the group consisting of a Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide. The remaining (non-endogenously expressed) proteins from the group can then be heterologously expressed in the cells, and screened for re-programming and/or de-differentiation into pluripotent cells, optionally in the presence of one or more of a MEK inhibitor, an agent that inhibits H3K9 methylation, an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear recep-

tor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; and/or an Erk inhibitor.

It is believed that any type of mammalian non-pluripotent cell can be screened for protein expression and subsequently be converted to a pluripotent cell. In some embodiments, the starting cells are isolated progenitor cells. Exemplary progenitor cells include, but are not limited to, endoderm progenitor cells, mesoderm progenitor cells (e.g., muscle progenitor cells, bone progenitor cells, blood progenitor cells), and ectoderm progenitor cells (e.g., epidermal tissue progenitor cells and neural progenitor cells). Cells useful for these aspects of the invention can be readily identified by screening cell lines for expression of a Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide or by identifying cells with reduced promoter methylation (e.g., by DNA bisulfite sequencing) or identifying cells with a modified histone state to determine the reduced silencing state of these genes. Those transcription factors that are not expressed endogenously can then be expressed heterologously to induce pluripotency without heterologous expression of those factors already expressed endogenously.

As shown in the Examples, some cells (e.g., neural progenitor cells and fibroblasts) can be induced into pluripotency by heterologous expression of Oct4 and Klf4 only. This demonstrates that Sox and Myc proteins, and likely Oct and Klf proteins, need not all be overexpressed (e.g., using a high expression viral vector) to achieve pluripotency. Instead, some of these proteins can be expressed at endogenous or even lower detectable levels and still be eligible for conversion to pluripotent cells by heterologous expression of other members of the group. Thus, in some embodiments of the invention, cells are identified that endogenously express a Sox polypeptide and/or a Myc polypeptide, and an Oct polypeptide and a Klf polypeptide is heterologously expressed in the cell, thereby inducing conversion of the cell into a pluripotent cell. In some embodiments, cells are identified that endogenously express an Oct polypeptide and/or a Klf polypeptide and/or a Myc polypeptide, and a Sox polypeptide is heterologously expressed in the cell, thereby inducing conversion of the cell into a pluripotent cell. Optionally, Sal14 (Zhang et al., *Nat Cell Biol.* 8(10): 1114-23 (2006)) can be expressed in place of any or all of Myc, Klf4, and Sox2.

Efficiency of induction to pluripotency as described herein can be further improved by inclusion in non-pluripotent cells of, e.g., one or more of, UTF1, SV40, TERT (either by introduction of an expression cassette encoding these gene products, or by contacting the cells with the proteins themselves) and/or by reducing expression of p53 (e.g., by siRNA). See, e.g., Zhao, et al., *Cell Stem Cell* 3:475-479 (2008).

C. Transcription Factor Proteins

As detailed herein, a number of embodiments of the invention involve introduction of one or more polypeptides into cells, thereby inducing pluripotency in the cell. Introduction of a polypeptide into a cell can comprise introduction of a polynucleotide comprising one or more expression cassettes into a cell and inducing expression, thereby introducing the polypeptides into the cell by transcription and translation from the expression cassette. Alternatively, one can introduce an exogenous polypeptide (i.e., a protein provided from outside the cell and/or that is not produced by the cell) into the cell by a number of different methods that do not involve introduction of a polynucleotide encoding the polypeptide.

Accordingly, for any embodiment of the invention described herein referring either to introduction of a polypeptide into a cell, or introduction of an expression cassette encoding a polypeptide into a cell, it should be understood that the present invention also expressly provides for exogenous introduction of the polypeptide as a protein into the cell. Therefore, in some embodiments, mammalian non-pluripotent cells are induced to pluripotency by a) exogenously introducing one or more of a Klf polypeptide, an Oct polypeptide, a Myc polypeptide, and/or a Sox polypeptide into the non-pluripotent cells; and optionally b) contacting the cells with one or more of a MEK inhibitor, an agent that inhibits H3K9 methylation, an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor, thereby producing induced pluripotent stem cells.

In some embodiments, in some embodiments of the invention, non-pluripotent cells are identified that endogenously express at least one (and optionally, two or three of) proteins from the group consisting of a Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide. The remaining (non-endogenously expressed) proteins from the group can then be exogenously introduced in the cells, and screened for re-programming and/or de-differentiation into pluripotent cells, optionally in the presence of one or more of a MEK inhibitor, an agent that inhibits H3K9 methylation, an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor.

In some embodiments, cells are identified that endogenously express a Sox polypeptide and/or a Myc polypeptide, and as a second step an Oct polypeptide and a Klf polypeptide is exogenously introduced in the cell, thereby inducing conversion of the cell into a pluripotent cell. In some embodiments, cells are identified that endogenously express an Oct polypeptide and/or a Klf polypeptide and/or a Myc polypeptide, and a Sox polypeptide is exogenously introduced in the cell, thereby inducing conversion of the cell into a pluripotent cell.

Exogenous introduction of a polypeptide into a cell can occur in any number of ways. One or more proteins can simply be cultured in the presence of target cells under conditions to allow for introduction of the proteins into the cell. In some embodiments, the exogenous proteins comprise the transcription factor polypeptide of interest linked (e.g., linked as a fusion protein or otherwise covalently or non-covalently linked) to a polypeptide that enhances the ability of the transcription factor to enter the cell (and optionally the cell nucleus).

Examples of polypeptide sequences that enhance transport across membranes include, but are not limited to, the *Drosophila* homeoprotein antennapedia transcription protein (AntHD) (Joliot et al., *New Biol.* 3: 1121-34, 1991; Joliot et al., *Proc. Natl. Acad. Sci. USA,* 88: 1864-8, 1991; Le Roux et al., *Proc. Natl. Acad. Sci. USA,* 90: 9120-4, 1993), the herpes simplex virus structural protein VP22 (Elliott and O'Hare, Cell 88: 223-33, 1997); the HIV-1 transcriptional activator TAT protein (Green and Loewenstein, Cell 55: 1179-1188, 1988; Frankel and Pabo, Cell 55: 1 289-1193, 1988); delivery enhancing transporters such as described in U.S. Pat. No. 6,730,293 (including but not limited to an peptide sequence comprising at least 7-25 contiguous arginines); and commercially available Penetra-

tin™ 1 peptide, and the Diatos Peptide Vectors ("DPVs") of the Vectocell® platform available from Daitos S. A. of Paris, France. See also, WO/2005/084158 and WO/2007/123667 and additional transporters described therein. Not only can these proteins pass through the plasma membrane but the attachment of other proteins, such as the transcription factors described herein, is sufficient to stimulate the cellular uptake of these complexes.

In some embodiments, the transcription factor polypeptides described herein are exogenously introduced as part of a liposome, or lipid cocktail such as commercially available Fugene6 and Lipofectamine). In another alternative, the transcription factor proteins can be microinjected or otherwise directly introduced into the target cell.

As discussed in the Examples, incubation of cells with the transcription factor polypeptides of the invention for extended periods can be toxic to the cells. Therefore, the present invention provides for intermittent incubation of non-pluripotent mammalian cells with one or more of Klf polypeptide, an Oct polypeptide, a Myc polypeptide, and/or a Sox polypeptide, with intervening periods of incubation of the cells in the absence of the one or more polypeptides. In some embodiments, the cycle of incubation with and without the polypeptides can be repeated for 2, 3, 4, 5, 6, or more times and is performed for sufficient lengths of time (i.e., the incubations with and without proteins) to achieve the development of pluripotent cells. Various agents (e.g., MEK inhibitor and/or GSK inhibitor and/or TGFbeta inhibitor) can be included to improve efficiency of the method.

III. Non-Pluripotent Cells

As used herein, "non-pluripotent cells" refer to mammalian cells that are not pluripotent cells. Examples of such cells include differentiated cells as well as progenitor cells. Examples of differentiated cells include, but are not limited to, cells from a tissue selected from bone marrow, skin, skeletal muscle, fat tissue and peripheral blood. Exemplary cell types include, but are not limited to, fibroblasts, hepatocytes, myoblasts, neurons, osteoblasts, osteoclasts, and T-cells.

In some embodiments where an individual is to be treated with the resulting pluripotent cells, the individual's own non-pluripotent cells are used to generate pluripotent cells according to the methods of the invention.

Cells can be from, e.g., humans or non-human mammals. Exemplary non-human mammals include, but are not limited to, mice, rats, cats, dogs, rabbits, guinea pigs, hamsters, sheep, pigs, horses, and bovines.

IV. Transformation

This invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook et al., *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel et al., eds., 1994)).

In some embodiments, the species of cell and protein to be expressed is the same. For example, if a mouse cell is used, a mouse ortholog is introduced into the cell. If a human cell is used, a human ortholog is introduced into the cell.

It will be appreciated that where two or more proteins are to be expressed in a cell, one or multiple expression cassettes can be used. For example, where one expression cassette is to express multiple polypeptides, a polycistronic expression cassette can be used.

In some embodiments, expression cassettes encoding reprogramming transcription factor proteins can be introduced into cells as part of a vector. Exemplary vectors include, e.g., plasmids, and viral vectors (including but not limited to adenoviral, AAV, or retroviruses such as lentiviruses).

V. Culturing of Cells

Cells to be induced to pluripotency can be cultured according to any method known in the art. General guidelines can be found in, e.g., Maherali, et al., *Cell Stem Cell* 3:595-605 (2008).

In some embodiments, the cells are cultured in contact with feeder cells. Exemplary feeder cells include, but are not limited to fibroblast cells, e.g., mouse embryonic fibroblast (MEF) cells. Methods of culturing cells on feeder cells is known in the art.

In some embodiments, the cells are cultured in the absence of feeder cells. Cells, for example, can be attached directly to a solid culture surface (e.g., a culture plate), e.g., via a molecular tether. The inventors have found that culturing cells induced to pluripotency have a much greater efficiency of induction to pluripotency (i.e., a greater portion of cells achieve pluripotency) when the cells are attached directly to the solid culturing surface compared the efficiency of otherwise identically-treated cells that are cultured on feeder cells. Exemplary molecular tethers include, but are not limited to, matrigel, an extracellular matrix (ECM), ECM analogs, laminin, fibronectin, or collagen. Those of skill in the art however will recognize that this is a non-limiting list and that other molecules can be used to attach cells to a solid surface. Methods for initial attachment of the tethers to the solid surface are known in the art.

As used in this "culturing" section, "cells to be induced to pluripotency" are induced by any method in the art, including, but not limited to the methods described in this application.

VI. Uses for Pluripotent Cells

The present invention allows for the further study and development of stem cell technologies, including but not limited to, prophylactic or therapeutic uses. For example, in some embodiments, cells of the invention (either pluripotent cells or cells induced to differentiate along a desired cell fate) are introduced into individuals in need thereof, including but not limited to, individuals in need of regeneration of an organ, tissue, or cell type. In some embodiments, the cells are originally obtained in a biopsy from an individual; induced into pluripotency as described herein, optionally induced to differentiate (for examples into a particular desired progenitor cell) and then transplanted back into the individual. In some embodiments, the cells are genetically modified prior to their introduction into the individual.

In some embodiments, the pluripotent cells generated according to the methods of the invention are subsequently induced to form, for example, hematopoietic (stem/progenitor) cells, neural (stem/progenitor) cells (and optionally, more differentiated cells, such as subtype specific neurons, oligodendrocytes, etc), pancreatic cells (e.g., endocrine progenitor cell or pancreatic hormone-expressing cells), hepato-

cytes, cardiovascular (stem/progenitor) cells (e.g., cardiomyocytes, endothelial cells, smooth muscle cells), retinal cells, etc.

A variety of methods are known for inducing differentiation of pluripotent stem cells into desired cell types. A non-limiting list of recent patent publications describing methods for inducing differentiation of stem cells into various cell fates follows: U.S. Patent Publication No. 2007/0281355; 2007/0269412; 2007/0264709; 2007/0259423; 2007/0254359; 2007/0196919; 2007/0172946; 2007/0141703; 2007/0134215.

A variety of diseases may be ameliorated by introduction, and optionally targeting, of pluripotent cells of the invention to a particular injured tissue. Examples of disease resulting from tissue injury include, but are not limited to, neurodegeneration disease, cerebral infarction, obstructive vascular disease, myocardial infarction, cardiac failure, chronic obstructive lung disease, pulmonary emphysema, bronchitis, interstitial pulmonary disease, asthma, hepatitis B (liver damage), hepatitis C (liver damage), alcoholic hepatitis (liver damage), hepatic cirrhosis (liver damage), hepatic insufficiency (liver damage), pancreatitis, diabetes mellitus, Crohn disease, inflammatory colitis, IgA glomerulonephritis, glomerulonephritis, renal insufficiency, decubitus, burn, sutural wound, laceration, incised wound, bite wound, dermatitis, cicatricial keloid, keloid, diabetic ulcer, arterial ulcer and venous ulcer.

The agents described herein (e.g., an agent that inhibits H3K9 methylation; an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor) are useful therapeutic agents alone, or in combination with each other as described herein. For example, the agents, or combinations thereof, are useful for reducing tissue damage and thus can be administered to treat, ameliorate, or prevent tissue damage. In some embodiments, an agent of the invention is administered to an individual having, or at risk of having tissue damage to an internal organ. Internal organs include, but are not limited to, brain, pancreas, liver, intestine, lung, kidney, or heart, wounding, e.g., by burn or cut. For example, in some embodiments, the agents of the invention are effective in reducing infarction size in reperfusion following ischemia. Thus, an agent of the invention can be administered to individuals at risk of having, having, or who have had, a stroke. Similarly, an agent of the invention can be administered to individuals at risk of having, having, or who have had, a heart attack or cardiac damage.

Active compounds described herein also include the salts, hydrates, solvates and prodrug forms thereof. The compounds of the present invention also include the isomers and metabolites thereof. Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are all intended to be encompassed within the scope of the present invention. For example, the compound of the present invention can be the R-isomer or the S-isomer, or a mixture thereof. In addition, the compound of the present invention can be the E-isomer or the Z-isomer, or a combination thereof.

Pharmaceutically acceptable salts of the acidic compounds of the present invention are salts formed with bases, namely cationic salts such as alkali and alkaline earth metal salts, such as sodium, lithium, potassium, calcium, magnesium, as well as ammonium salts, such as ammonium, trimethyl-ammonium, diethylammonium, and tris-(hydroxymethyl)-methyl-ammonium salts. In some embodiments, the present invention provides the hydrochloride salt. In other embodiments, the compound is ellipticine hydrochloride.

Similarly acid addition salts, such as of mineral acids, organic carboxylic and organic sulfonic acids, e.g., hydrochloric acid, methanesulfonic acid, maleic acid, are also possible provided a basic group, such as pyridyl, constitutes part of the structure.

The neutral forms of the compounds can be regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound can differ from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

The compounds of the present invention can be made by a variety of methods known to one of skill in the art (see *Comprehensive Organic Transformations* Richard C. Larock, 1989). One of skill in the art will appreciate that other methods of making the compounds are useful in the present invention.

Administration of cells or compounds described herein is by any of the routes normally used for introducing pharmaceuticals. The pharmaceutical compositions of the invention may comprise a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., *Remington's Pharmaceutical Sciences,* 17th ed. 1985)).

Formulations suitable for administration include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, orally, nasally, topically, intravenously, intraperitoneally, intrathecally or into the eye (e.g., by eye drop or injection). The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials. Solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. The modulators can also be administered as part of a prepared food or drug.

The dose administered to a patient, in the context of the present invention should be sufficient to induce a beneficial response in the subject over time, i.e., to ameliorate a condition of the subject. The optimal dose level for any patient will depend on a variety of factors including the efficacy of the specific modulator employed, the age, body weight, physical activity, and diet of the patient, and on a possible combination with other drug. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular compound or vector in a particular subject. Administration can be accomplished via single or divided doses.

VII. Screening for Agents that Induce Pluripotent Stem Cell Development

The present invention provides for methods of screening for agents that can "replace" one of the four iPS transcription

factors (i.e., an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide), or alternatively can replace an Oct polypeptide, a Klf polypeptide, or a Sox polypeptide in cells where Myc is not necessary to reprogram cells into pluripotent cells (Nakagawa, M. et al. *Nature Biotechnol.* 26, 101-106 (2007); Wernig, M., Meissner, A., Cassady, J. P. & Jaenisch, R. *Cell Stem Cell* 2, 10-12 (2008)) or alternatively improve the efficiency of induction to pluripotency.

In some embodiments, the methods comprise introducing one or more expression cassettes for expression of at least one of, but not all of, an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide into non-pluripotent cells to generate transfected cells; subsequently contacting the transfected cells to a library of different agents; screening the contacted cells for pluripotent stem cell characteristics; and correlating the development of stem cell characteristics with a particular agent from the library, thereby identifying an agent that stimulates dedifferentiation of cells into pluripotent stem cells. In some embodiments, the cells are contacted with at least one of an agent that inhibits H3K9 methylation; an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor as well as one or more members of a small molecule or other agent library to identify a library member that induces or improves induction of cells to pluripotency. Thus, mixtures of non-pluripotent cells and at least one (e.g., 1, 2, 3, 4, 5 or more of) an agent that inhibits H3K9 methylation; an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor are provided in the present invention.

The agents in the library can be any small chemical compound, or a biological entity, such as a protein, sugar, nucleic acid or lipid. Typically, test agents will be small chemical molecules and peptides. Essentially any chemical compound can be used as a potential agent in the assays of the invention, although most often compounds that can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, Mo.), Aldrich (St. Louis, Mo.), Sigma-Aldrich (St. Louis, Mo.), Fluka Chemika-Biochemica Analytika (Buchs, Switzerland) and the like.

In some embodiments, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential iPS replacement agents (potentially acting to replace one of the iPS proteins). Such "combinatorial chemical libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity, i.e., such as inducing pluripotent stem cell characteristics in cells that express some, but not all of, an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Pat. No. 5,010,175, Furka, Int. J. Pept. Prot. Res. 37:487-493 (1991) and Houghton et al., *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs et al., *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara et al., *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann et al., *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen et al., *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho et al., *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell et al., *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (see Ausubel, Berger and Sambrook, all supra), peptide nucleic acid libraries (see, e.g., U.S. Pat. No. 5,539,083), antibody libraries (see, e.g., Vaughn et al., *Nature Biotechnology,* 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (see, e.g., Liang et al., *Science,* 274: 1520-1522 (1996) and U.S. Pat. No. 5,593,853), small organic molecule libraries (see, e.g., benzodiazepines, Baum C&EN, January 18, page 33 (1993); isoprenoids, U.S. Pat. No. 5,569,588; thiazolidinones and metathiazanones, U.S. Pat. No. 5,549,974; pyrrolidines, U.S. Pat. Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Pat. No. 5,506, 337; benzodiazepines, U.S. Pat. No. 5,288,514, and the like).

Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville Ky., Symphony, Rainin, Woburn, Mass., 433A Applied Biosystems, Foster City, Calif., 9050 Plus, Millipore, Bedford, Mass.). In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Tripos, Inc., St. Louis, Mo., 3D Pharmaceuticals, Exton, Pa., Martek Biosciences, Columbia, Md., etc.).

Cells contacted with the agents, and optionally expressing some but not all of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide (e.g., combinations of one, two or three of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide), can then be screened for the development of pluripotent cells, e.g., by screening for one or more pluripotent stem cell characteristics. Initial screens can be designed by transforming the cells to be screened with an expression cassette comprising promoter elements known to be activated in pluripotent stem cells (optionally, but not other cells) operably linked to a selectable or otherwise identifiable marker. For example, a detectable marker such as GFP or other reporter system can be used. Exemplary promoter elements known to be activated in pluripotent cells include, but are not limited to,

Oct4, Nanog, SSEA1 and ALP promoter sequences. Cells can also be screened for expression of other pluripotent cell markers (e.g., by immunofluorescence, etc.) as are known in the art, including, but not limited to Nanog, SSEA1 and ALP. In some embodiments, cell morphology is examined.

In some embodiments, the cells are cultured in the presence of a MAPK/ERK kinase (MEK) inhibitor. The inventors have found that the presence of a MEK inhibitor results in both inhibition of growth of non-pluripotent cells and stimulation of growth of pluripotent stem cells. This effect therefore magnifies the "signal" of the screen and allows for more efficient and sensitive detection of agents that induce reprogramming of cells into pluripotent stem cells. A wide variety of MEK inhibitors are known, including but not limited to, PD0325901 (see, e.g., Thompson, et al., *Current Opinion in Pharmacology* 5(4): 350-356 (2005)); MEK Inhibitor U0126 (Promega), ARRY-886 (AZD6244) (Array Biopharma); PD98059 (Cell Signaling Technology); and Amino-thio-acrylonitriles (U.S. Pat. No. 6,703,420). Other MEK inhibitors are described in, e.g., U.S. Pat. No. 6,696,440 and WO 04/045617, among others.

IX. Cell Mixtures and Media

As discussed herein, the present invention provides for non-pluripotent cells in a mixture with one or more compound selected from the group consisting of an agent that inhibits H3K9 methylation; an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a histone deacetylase (HDAC) inhibitor; or an Erk inhibitor. In some embodiments, the compound is in the mixture at a concentration sufficient to induce or improve efficiency of induction to pluripotency. For example, in some embodiments, the compounds are in a concentration of at least 0.1 nM, e.g., at least 1, 10, 100, 1000, 10000, or 100000 nM, e.g., between 0.1 nM and 100000 nM, e.g., between 1 nM and 10000 nM, e.g., between 10 nM and 10000 nM. In some embodiments, the mixtures are in a synthetic vessel (e.g., a test tube, Petri dish, etc.). Thus, in some embodiments, the cells are isolated cells (not part of an animal). In some embodiments, the cells are isolated from an animal (human or non-human), placed into a vessel, contacted with one or more compound as described herein. The cells can be subsequently cultured and optionally, inserted back into the same or a different animal, optionally after the cells have been stimulated to become a particular cell type or lineage.

In some embodiments, the present invention provides a eukaryotic cell medium comprising a TGFβ receptor/ALK5 inhibitor (including but not limited to SB431542 or A-83-01). In some embodiments, the cell medium comprises a TGFβ receptor/ALK5 inhibitor and an agent selected from the group consisting of a MEK inhibitor (including but not limited to PD0325901) and an Erk inhibitor (including but not limited to PD98059). In some embodiments, the cell medium comprises a TGFβ receptor/ALK5 inhibitor and a GSK-3 inhibitor (including but not limited to CHIR99021). In some embodiments, the cell medium of the present invention comprises a TGFβ receptor/ALK5 inhibitor, a GSK-3 inhibitor, and an agent selected from the group consisting of a MEK inhibitor and an Erk inhibitor. Further, the present invention provides a eukaryotic cell medium comprising a GSK-3 inhibitor (including but not limited to CHIR99021). In some embodiments, the cell medium of the present invention comprises a GSK-3 inhibitor and an agent that inhibits H3K9 methylation or promotes H3K9 demethylation (including but not limited to BIX01294).

In some embodiments, the cell medium of the invention further comprises an agent that inhibits H3K9 methylation or promotes H3K9 demethylation, an L-type Ca channel agonist, an activator of the cAMP pathway, DNA methyltransferase (DNMT) inhibitor, a nuclear receptor ligand, a histone deacetylase (HDAC) inhibitor, an erk inhibitor, or a histone methyltransferase (HMT) inhibitor. For example, in some cases, the eukaryotic cell medium comprises a TGFβ receptor/ALK5 inhibitor and a histone deacetylase (HDAC) inhibitor (e.g., sodium butyrate or valproic acid). In some embodiments, the cell medium comprises a TGFβ receptor/ALK5 inhibitor, a MEK inhibitor or an Erk inhibitor, and a histone deacetylase (HDAC) inhibitor.

In some embodiments, the eukaryotic cell medium of the invention further comprises one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide. In some embodiments, the cell medium does not comprise cells. In some embodiments, the cell medium further comprises cells, e.g., non-pluripotent cells.

As explained herein, in some embodiments, the cells comprise an expression cassette for heterologous expression of at least one or more of an Oct polypeptide, a Myc polypeptide, a Sox polypeptide and a Klf polypeptide. In some embodiments, the cells do not include an expression cassette to express any of the Oct, Myc, Sox of Klf polypeptides. Cells with or without such expression cassettes are useful, for example, in screening methods as described herein.

Examples of non-pluripotent cells include those described herein, including but not limited to, cells from a tissue selected from bone marrow, skin, skeletal muscle, fat tissue and peripheral blood. Exemplary cell types include, but are not limited to, fibroblasts, hepatocytes, myoblasts, neurons, osteoblasts, osteoclasts, and T-cells.

The present invention also provides mixtures (with, or optionally without cells) of an agent that inhibits H3K9 methylation (including but not limited to BIX-01294) with a compound selected from at least one of an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor. In some including but not limited to embodiments, the agent and at least one compound listed above is at a concentration as described above. Such mixtures are useful, for example, as "pre-mixes" for induction of pluripotency in cells.

X. Kits

The present invention also provides kits, e.g., for use in inducing or improving efficiency of induction of pluripotency in cells. Such kits can comprise one of more compound selected from the group consisting of an agent that inhibits H3K9 methylation; an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor. In some embodiments, the kits comprise an agent that inhibits H3K9 methylation (including but not limited to BIX-01294) and a second compound (separate or mixed with agent that inhibits H3K9 methylation) selected from at least one of an L-type Ca channel agonist; an activator of the cAMP pathway; a DNA methyltransferase (DNMT) inhibitor; a nuclear receptor

ligand; a GSK3 inhibitor; a MEK inhibitor, a TGFβ receptor/ALK5 inhibitor, a HDAC inhibitor; or an Erk inhibitor.

The present invention further provides kits comprising a TGFβ receptor/ALK5 inhibitor (including but not limited to SB431542 or A-83-01), e.g., for use in inducing pluripotency in non-pluripotent cells or improving efficiency of induction of pluripotency in cells. In some embodiments, such kits comprise a TGFβ receptor/ALK5 inhibitor and an agent selected from the group consisting of a MEK inhibitor (including but not limited to PD0325901) and an Erk inhibitor (including but not limited to PD98059). The present invention further provides kits comprising a GSK-3 inhibitor (including but not limited to CHIR99021), e.g., for use in inducing pluripotency in non-pluripotent cells or improving efficiency of induction of pluripotency in cells. One or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide and/or polynucleotide encoding one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide can be included in combination with a TGFβ receptor/ALK5 inhibitor. Further, such kits can further comprise an agent that inhibits H3K9 methylation or promotes H3K9 demethylation (including but not limited to BIX01294), an L-type Ca channel agonist, an activator of the cAMP pathway, DNA methyltransferase (DNMT) inhibitor, a nuclear receptor ligand, a histone deacetylase (HDAC) inhibitor, an erk inhibitor, or a histone methyltransferase (HMT) inhibitor. In some embodiments, the agent is a histone deacetylase (HDAC) inhibitor (e.g., sodium butyrate or valproic acid).

In some embodiments, the kits further comprise non-pluripotent cells. Examples of non-pluripotent cells include those described herein, including but not limited to, cells from a tissue selected from bone marrow, skin, skeletal muscle, fat tissue and peripheral blood. Exemplary cell types include, but are not limited to, fibroblasts, hepatocytes, myoblasts, neurons, osteoblasts, osteoclasts, and T-cells.

EXAMPLES

Example 1

Toward identifying conditions that can replace viral transduction of oncogenic transcription factors (e.g. cMyc and Oct4 (Hochedlinger, K. et al., *Cell* 121, 465-477 (2005)) and enhance reprogramming efficiency, we sought to exploit combination of two approaches: one was to examine a defined progenitor cell type based on the notion that certain accessible adult progenitor cells may endogenously express at certain level some of the required genes for inducing pluripotency and/or the loci of these genes may be less silenced so that such progenitor cells might be more efficiently reprogrammed and/or with less genetic manipulations; the other approach was to screen small molecules that may be able to replace viral integration of specific transcription factor and/or promote reprogramming process.

Among various adult stem/progenitor cells that are accessible from different tissues, we initially focused our efforts on neural progenitor cells for the following reasons: (i) In contrast to heterogeneous primary fibroblast culture (e.g., MEF) which may contain various types of stem/progenitor cells, neural progenitor cells are a relatively defined population of cells and can be clonally expanded under chemically defined conditions. (ii) Neural progenitor cells endogenously express specific Sox genes (e.g. Sox1 or Sox2), which, although is at lower level than overexpression, might be sufficient for generating iPS cells. (iii) Neural progenitor cells or Sox gene expressing cells may be isolated from other tissues (Fernandes, K. J. L. et al., *Nature Cell Biology* 6, 1082-1093 (2004); Seaberg, R. M. et al., *Nature Biotechnol.* 22, 1115-1124 (2004)) and expanded in vitro. Therefore, defined neural progenitor cells represent an excellent model system to address above questions in reprogramming process/mechanism. To establish an unlimited, highly reproducible and defined source of neural progenitor cells that can be used in high throughput screens, we chose to use mESC-derived neural progenitor cells that contain a GFP-IRES-Puro/GiP reporter under control of the Oct4 regulatory elements, since mESCs can be grown in large quantity and their differentiation to a homogenous population of neural progenitor cells is well defined (Conti, L. et al., *PLoS Biol.* 3, e283 (2005)), and the validated reporter activity (Ying, Q. L. et al., *Nature* 416, 545-548 (2002)) can facilitate facile assay detection.

The reporter neural progenitor cells were generated using a well established protocol by differentiating the Oct4-GiP mESCs grown in monolayer on gelatin in a chemically defined medium/CDM condition in the absence of serum and other growth factors/cytokines at low cell density for eight days, followed by neurophere formation and subsequent serial passaging in single cells in neural cell expansion media supplemented with 10 ng/ml of bFGF and EGF in monolayer for over six passages/24 days. The resulting neural progenitor cells were homogenous by cell morphology and neural marker expression, and were confirmed to be GFP negative and puromycin sensitive. Such neural progenitor cells plated in monolayer in conventional mESC growth media were transduced with combinations of four, three, or two of the four factors, followed by treating the transduced cells with individual small molecules from a small known drug collection in a typical 6-well format. The compound treatment and culture were continued for additional ten days before puromycin was added. The number of green and puro-resistant colonies was counted at day 14. In comparison to the only four-gene transduced neural cells as the positive control, compound conditions that generated more green colonies than the corresponding gene-only conditions were picked as primary hits. To further confirm these primary hit conditions, we chose to use a late passage of mouse CNS neural progenitor cells (Do, J. T. et al., *Stem Cells* 25, 1013-1020 (2007)) that were derived from fetal brain of $OG2^{+/-}/ROSA26^{+/-}$ transgenic mice (which contain an Oct4-GFP reporter) and expanded in monolayer under the same neural CDM condition as the above with 10 ng/ml of bFGF and EGF. Such cells truly from a non-pluripotent tissue would be devoid of concerns of any contaminating ES cell in the above screening system, which is highly unlikely with all appropriate controls. Similar culture conditions and reprogramming assays were performed using the OG2 neural progenitor cells except that puromycin was not used and green colonies were picked out and characterized by staining of Nanog, SSEA1 and ALP. We found that almost all of the green colonies that can be identified at day 12-14 can be expanded to long-term stable iPS cells that are indistinguishable from the classic mESCs by morphology and typical pluripotency marker expression.

We first focused our characterization efforts on two new conditions that could be reconfirmed using the fetal neural progenitor cells. Just as hypothesized that certain tissue-specific progenitor cells with endogenous expression of certain relevant reprogramming genes may require less exogenous genetic manipulation to generate iPS cells, we found that viral transduction of only Oct4 and Klf4 together is sufficient to generate iPS cells from neural progenitor cells in 10-14 days. While such reprogramming efficiency (1-2

GFP colonies per 3.5×10^4 cells) is lower than conditions with additional Sox2 and cMyc viral transduction (8-10 GFP colonies per 3.5×10^4 cells) (FIG. **1**), it is interesting that the reprogramming kinetics by the two genes only (Oct4 and Klf4) is not significantly slower than that by the original four genes. This is in contrast to the recent observations that omitting cMyc in generating iPS cells from MEFs is significantly slower (e.g. additional 2 weeks) than the condition having cMyc overexpression even the embryonic fibroblasts endogenously express cMyc. Most interestingly, we found that a small molecule, BIX01294 (Kubicek, S. et al., *Molecular Cell* 25, 473-481 (2007)) that specifically inhibits G9a (a hi stone methyltransferase for H3K9me2), can significantly improve the reprogramming efficiency to or higher than the level of using viral transduction of all four factors, while it didn't significantly shorten the kinetics of reprogramming. The reprogramming event is typically assayed by the ability of identifying the iPS cell colonies, which is influenced by many factors, including the methods of cell culture, cell identification and/or selection, as well as the input cell type, number, and reprogramming efficiency and kinetics. Consequently, a requirement for any given gene for reprogramming is relative to that specific setting and largely depends on the reprogramming efficiency/kinetics. In this regard, this single small molecule, BIX01294, functionally replaced viral transduction of cMyc and Sox2 to large extent.

GFP+ iPS cell colonies readily appeared 12 days after OG2 neural progenitor cells were transduced with Oct4/Klf4 retroviruses and treated with BIX01294. Day 14 iPS cells generated from Oct4-Klf4 viral transduction and BIX01294 treatment can be readily expanded in the conventional mESC culture condition on MEF feeder cells in the presence of LIF without the requirement of continued BIX01294 treatment. iPS cells generated by Oct4/Klf4 viral transduction and BIX01294 treatment can long-term self-renew on MEF feeders in the mESC growth media without continued BIX01294 treatment. They grow as compact and domed colonies. These iPS cells maintain characteristic mESC-colony morphology, homogenously express typical pluripotency markers in comparable level as mESCs, including Oct4, Nanog, SSEA1 and ALP by immunocytochemistry, histostaining and RT-PCR analysis. Furthermore, such iPS cells, which had been serially passaged over 10 passages, can effectively differentiate into characteristic neurons (βIII-tubulin), beating cardiomyocytes (cardiac troponin) and pancreatic or hepatic cells (Pdx1 or Albumin), derivatives of the three primary germ layers under standard embryoid body or directed differentiation methods. And most importantly, such iPS cells can efficiently incorporate into the ICM of a blastocyst after aggregation with an 8-cell embryo, lead to a high-grade chimerism after the aggregated embryos were transplanted into mice, and contribute to the germ line in vivo. These in vitro and in vivo characterizations confirm that the iPS cells generated by Oct4 and Klf4 viral transduction with simultaneous BIX01294 treatment are morphologically, functionally and by typical pluripotency marker expression indistinguishable from the original four-factor iPS cells and the classic mESCs.

One question is whether expression of Oct4, Sox2, Klf4 and cMyc, regardless of endogenously or exogenously, would be a prerequisite for generating iPS cells. Interestingly, recent reprogramming studies on generating human iPS cells from fibroblasts have shown while exogenous expression of Klf4 and cMyc are functionally exchangeable with Nanog and Lin28, expression of Oct4 and Sox2 seems to be required so far from all published iPS cell studies. Interestingly, we found that viral transduction of Klf4, Sox2, and cMyc with simultaneous BIX01294 treatment in the absence of Oct4 expression can also generate iPS cells (FIG. **2**) while viral transduction of such three factors/KSM alone failed to produce any iPS cell colony under our assay conditions. Similarly, such KSM-BIX01294 generated iPS cells can be stably expanded and long-term self-renew on MEF feeders in the conventional mESC growth conditions over passages without BIX01294, maintain the characteristic mESC morphologies, homogenously express typical pluripotency markers, including ALP, Oct4, Nanog and SSEA1, and differentiate into cells in the three germ layers in vitro. It should be noted that the reprogramming efficiency in the absence of Oct4 expression is relatively low.

Finally, we observed that the application of a specific small molecule inhibitor of MEK, PD0325901, to late stage of reprogramming (e.g. after Oct4-GFP activation) can serve as an excellent selection strategy for generating iPS cells. Due to the very low efficiency of reprogramming, iPS cells are typically selected out by utilizing reporter (e.g. Neo/Puro or GFP) that is under control of the regulatory elements of a pluripotency marker using genetically modified somatic cell lines, or manually picked out based on cell morphology. While the later method applicable to genetically unmodified cells is better suited for ultimate clinical application of iPS cells, it is a much more tedious and less reliable technique that typically requires picking and propagating over several passages many colonies, only a small fraction of which would become true iPS cells efficiently. This is partly because that large percentage of similarly looking colonies may be partially reprogrammed cells and/or simply transformed cells, which grow rapidly and may interfere with growth and reprogramming of iPS cells. Consequently, having an alternative selection strategy for genetically unmodified cells would be highly desirable. We found that PD0325901 inhibits growth of non-iPS cells while it effectively promotes growth and stable reprogramming of iPS cells, leading to larger and more homogenous colonies of iPS cells. This observation might be partly due to the mechanism that MEK activity is required for cell cycle progression of somatic cells, while mESCs lack of such restriction for growth and inhibition of MEK also inhibits differentiation of mESCs (contributing to further stabilization of the iPS cell state).

The results presented here have a number of important implications. (1) The lower endogenous expression level (than overexpression) of critical genes required for reprogramming by (tissue-specific progenitor) somatic cells may be sufficient to replace the corresponding exogenous gene expression via viral transduction for generating iPS cells. This points to an alternative strategy of generating iPS cells from somatic cells using less genetic manipulation by exploiting practically accessible cells that endogenously express certain relevant reprogramming genes via an intrinsic tissue specificity and/or ex vivo culture manipulation. (2) This is a proof-of-principle demonstration that small molecules can be identified from rationally designed cell-based screens to functionally replace viral transduction of certain transcription factor(s), improve reprogramming efficiency, or serve as a selection condition in generating iPS cells. Not only may such pharmacological approach for replacing specific genetic manipulation substantially reduce risks associated with insertion of oncogenes (e.g. cMyc and Oct4) and insertional mutagenesis, but also it opens up the possibility of enabling a precisely controlled and highly efficient reprogramming process by defined small molecules. This is especially important for studying the molecular mechanism

of reprogramming, which currently is largely intractable due to its very low efficiency and slow kinetics. (3) In contract to the gain-of-function approach in generating iPS cells, the highly effective use of those specific small molecule inhibitors suggests that loss-of-function of specific genes may be at least equally important and effective in generating iPS cells. More importantly, the function of BIX01294 defines a specific epigenetic mechanism/target, i.e. inhibition of G9a-mediated H3K9me2, in generating iPS cells. This is consistent with the previous findings that the repressive H3K9 methylation is associated with Oct4 inactivation during differentiation (Feldman, N. et al., *Nature Cell Biology* 8, 188-194 (2006)), and histone lysine methylation, although robust, is dynamic and regulated by HMTases and lysine demethylases. BIX01294 may function to facilitate shifting the epigenetic balance from a silenced state of Oct4 to an active transcription. (4) Exemplified by using MEK inhibitor for facile selection of iPS cells, exploiting the differences between somatic cells and ESCs by small molecules represents an alternative/attractive strategy for selecting iPS cells. Finally, it is conceivable that the strategies and small molecules reported here can be further explored for improved approaches and better mechanistic understanding of generating iPS cells, and combined with additional small molecules (that can replace the function of remaining transduced transcription factors and improve reprogramming) as well as other non-genetic methods (e.g. protein transduction) to ultimately allow generation of iPS cells in high efficiency in a completely chemically defined condition without any genetic modification.

Methods.

Neural Progenitor Cell Culture.

Neural progenitor cells were derived from mESCs or mouse fetal brains according to the protocol reported by Conti et al (Conti, L. et al., *PLoS Biol.* 3, e283 (2005)). Briefly, mESCs were plated onto a 0.1% gelatin-coated dish at 1×10^4 cells/cm^2 in the neural induction medium (50% DMEM/F12 basal medium, 50% Neurobasal medium, 0.5× N2, 0.5×B27, 1× Glutamax, 50 ug/ml BSA) and differentiated for 7-8 days. The formed neural rosettes were then trypsinized into single cells and replated into an Ultra-Low Attachment dish (Corning) to form neurosphere in the neural progenitor cell expansion medium (DMEM/F12, 1×N2, 10 ng/ml bFGF, 10 ng/ml EGF, 50 ug/ml BSA). After three days in suspension neurospheres were re-attached to a gelatin-coated dish and further differentiated for 4-6 days before they were further passaged in single cells and grown in monolayer on gelatin-coated dishes in the neural progenitor cell expansion medium for over 5-6 passages.

Neurospheres from brains of 12.5 to 16.5 dpc ROSA26/OG2 heterozygous fetuses were generated as previously described (Do, J. T. et al., *Stem Cells* 25, 1013-1020 (2007)). Briefly, the cortex was dissected, enzymatically dissociated, and passed through a 70 μm nylon mesh (Falcon; Franklin Lakes, N.J.). Neural cells were further purified by centrifugation in 0.9 M sucrose in 0.5×HBSS at 750 g for 10 min and in 4% BSA in EBSS solution at 200 g for 7 min. Such cells were further grown in suspension to form neurospheres and subsequently serially passaged in monolayer on gelatin-coated dishes in the neural progenitor cell expansion medium as described above. Animal experiments were approved and performed according to the Animal Protection Guidelines of the Government of Max Planck Society, Germany.

Retrovirus Transduction.

The murine cDNAs for Oct4, Klf4, Sox2 and c-Myc were cloned into the pMSCV retroviral vector and verified by sequencing. The pMX-based retroviral vectors were obtained from Addgene. The virus production and transduction were performed as described.

iPS Cell Induction from Neural Progenitor Cells.

mESC-derived or primary OG2 mouse neural progenitor cells were plated into Matrigel (1:50, BD Biosciences) coated 6-well plates at 3.5×10^4 cells/well in the neural progenitor cell expansion media. After one day these cells were transduced with retrovirus for overnight, and the medium was changed into the mESC growth media [DMEM, 5% FBS, 10% KSR, 1× non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM β-mercaptoethanol (Gibco) and 10^3 unit/ml LIF (Chemicon)] with or without BIX01294 (0.5-1 μM). GFP-positive iPS cell colonies appeared after 9-14 days, and were picked out and expanded on MEF feeder cells with the mESC growth media.

Characterization Assays.

ALP staining was performed as instructed by the Alkaline Phosphatase Detection Kit (Chemicon). Cells were fixed in 4% paraformaldehyde, washed three times by PBS, and then incubated in PBS containing 0.3% TritonX-100 (Sigma) and 10% normal donkey serum (Jackson ImmunoResearch) for 30 min at room temperature. The cells were then incubated with primary antibody overnight at 4° C.: mouse anti-Oct4 antibody, mouse anti-SSEA1 antibody (1:200, Santa Cruz), rabbit anti-Sox2 antibody (1:200, Chemicon), rabbit anti-Nanog antibody (AbCam), rabbit anti-Pdx1 (1:200, from Dr. C. Wright), mouse anti-βIII-tubulin antibody (1:500, Covance), mouse anti-cardiac troponin T (1:200, DSHB), rabbit anti-Albumin antibody (DAKO). After washing, cells were further incubated with secondary antibodies: Alexa Fluro555 donkey anti-mouse IgG or Alexa Fluro555 donkey anti-rabbit IgG (1:500, Invitrogen) for 30 min at RT. Nuclei were detected by DAPI (Sigma) staining. Images were captured by Nikon TE2000-U.

Aggregation of iPS Cells with Zona-Free Embryos.

iPS cells were aggregated with denuded post-compacted eight-cell stage embryos to obtain aggregate chimera. Eight-cell embryos (B6C3F1) flushed from females at 2.5 dpc were cultured in microdrops of KSOM medium (10% FCS) under mineral oil. Clumps of iPS cells (10-20 cells) after short treatment of trypsin were chosen and transferred into microdrops containing zona-free eight-cell embryos. Eight-cell embryos aggregated with iPS cells were cultured overnight at 37° C., 5% CO2. Aggregated blastocysts developed from eight-cell stage were transferred into one uterine horn of a 2.5 dpc pseudopregnant recipient.

Example 2

Somatic cells can be induced into pluripotent stem cells (iPSC) with a combination of four transcription factors, Oct4/Sox2/Klf4/c-Myc or Oct4/Sox2/Nanog/LIN28. This provides an enabling platform to obtain patient specific cells for various therapeutic and research applications. However, several problems remain for this approach to be therapeutically relevant due to drawbacks associated with efficiency and viral genome-integration. As explained above, neural progenitor cells (NPCs) transduced with Oct4/Klf4 can be reprogrammed into iPSCs. However, NPCs express Sox2 endogenously, possibly facilitating reprogramming in the absence of exogenous Sox2. In this study, we identified a small molecule combination, BIX-01294 and BayK8644, that enables reprogramming of Oct4/Klf4 transduced mouse embryonic fibroblasts, which do not endogenously express the factors essential for reprogramming. This study demon-

strates that small molecules identified through a phenotypic screen can compensate for viral transduction of critical factors, such as Sox2, and improve reprogramming efficiency.

This example is aimed to assess if small molecules can replace specific viral transduction to obtain iPSCs from a general cell lineage, in which none of the TFs deemed essential for reprogramming, Oct4, Sox2 and Klf4, are expressed. Hence, mouse embryonic fibroblasts (MEFs) were used. Finding a small molecule that could replace one of these TFs in the induction of MEF reprogramming might lead to the identification of general pathways involved in this process. Such chemical strategy might be more amenable for therapeutic application. Consequently, we screened a collection of known drugs to identify small molecules that can enable the generation of iPSCs from MEFs transduced with OK, and thus, could compensate for the lack of Sox2 overexpression. Through the different screens performed we identified that a combination of BIX with Bayk8644 (BayK), a L-channel calcium agonist (Schramm, M. et al., *Nature,* 303:535-537 (1983)) was one of the most effective. Bayk was of interest because it exerts its effect upstream in cell signaling pathways, and does not directly cause epigenetic modifications. It is likely that this type of molecule, such as BayK or activators of the Wnt signaling pathway (Marson, A. et al., *Cell Stem Cell,* 3:132-135 (2008)), can be exploited to induce reprogramming in a more specific manner than molecules acting directly at the epigenetic level causing DNA or histone modification. Some of these epigenetic modifiers have already been shown to facilitate the reprogramming process, such as BIX (Shi, Y. et al., *Cell Stem Cell,* 2:525-528 (2008)), valproic acid (Huangfu, D. et al., *Nat Biotechnol,* 26:795-797 (2008)) and 5' azacytidine (Mikkelsen, T. et al., *Nature,* 454:49-55 (2008)).

This present study demonstrates that small molecules identified through a phenotypic screen can be used to effectively compensate for the viral transduction of another critical iPSC TF, Sox2, which is not endogenously expressed in fibroblasts. Moreover, it highlights the important contribution that small molecule screens will eventually make to the discovery of new molecular targets and mechanisms involved in complicated biological processes such as reprogramming.

Results

Phenotypic Screen Leads to the Discovery of Small Molecules that Enable MEF Reprogramming when Transduced with Only Two TFs.

Unmodified MEFs derived from E13-14 embryos of the 129 mice were used for the initial screen. MEFs were plated on Matrigel at 3.5×10^4 cells/well of a 6-well plate and transduced with OK (retroviral vectors expressing Oct4 and Klf4) alone. Within 14-21 days, treated cells were assessed for the appearance of colonies that had the characteristic embryonic stem cell (ESC) colony morphology and were positive for the pluripotency marker alkaline phosphatase (ALP). Such OK-transduced cells generated only a few small non-compact colonies, which were weakly positive for ALP expression. These colonies initially appeared within 21 days after viral transduction and were difficult to expand. Therefore, such assay system offered a clean background for the identification of small molecules having desirable reprogramming inducing activity. Using this system, compounds from a library of around 2000 known small molecules (see Experimental Procedures below) were screened and were identified as hits when they induced the appearance of ESC colonies that were strongly positive for ALP within 14-21 days after treatment. This image-based method provided a more accurate assessment of reprogramming as compared to homogenous reporter-based assay. BIX appeared to have the strongest effect with reproducible induction of more than 1-2 compact ESC-like colonies with high ALP expression. We observed that when MEFs were treated with BIX after OK viral transduction, compact colonies with strong ALP expression could be readily detected within approximately 14-21 days. These cells were also positive for Nanog, Oct4 and SSEA-1 expression. This result, obtained with a more general cell type, which does not endogenously express any of the three essential reprogramming genes, further validates our previous observation that BIX has strong reprogramming inducing activity and inhibition of the G9a HMTase can facilitate reprogramming (Shi, Y. et al., *Cell Stem Cell,* 2:525-528 (2008)). However, the reprogramming efficiency in MEFs transduced with OK and treated with BIX was still low, about 2 colonies/3.5×10^4 cells, in comparison to the four factor-induced reprogramming of MEFs or the OK/BIX NPC reprogramming (Shi, Y. et al., *Cell Stem Cell,* 2:525-528 (2008)). Therefore, we conducted a second screen using a similar protocol, but where BIX was added to the basal media after OK viral transduction. This provided a more permissive platform to identify new small molecules that could further improve reprogramming efficiency. More importantly, this second screen could facilitate discovery of small molecules that impact reprogramming in a more specific manner, for example by acting on signal transduction pathways rather than on histone or DNA modifying enzymes. In this second screen, we again assayed the library of around 2000 known small molecules (see Experimental Procedures), and confirmed two compounds that were able to act in a synergistic manner with BIX to improve reprogramming based on the criteria of the screen. One example is RG108, a DNA methyltransferase (DMNT) inhibitor (Brueckner, B. et al., *Cancer Res,* 65:6305-6311 (2005)), which enhanced reprogramming of OK transduced MEFs in the presence of BIX (FIG. **3**). However, similarly to BIX, RG108 is known to impact the cells at a general epigenetic level, and another DNA methyltransferase inhibitor, 5-azacytidine has already been shown to enhance reprogramming (Mikkelsen, T. S. et al., *Nature,* 454:49-55 (2008)). Therefore, RG108 was not pursued further for this study. Instead, we focused our phenotypic and functional characterization on another small molecule that was identified in the second screen, BayK, an L-calcium channel agonist. This small molecule, which showed the strongest effect in the screen aside from known DNA/histone modifiers, was studied further because it has no observable reprogramming activity on OK-transduced MEFs in the absence of BIX and is not known to impact the cells directly at the epigenetic level, but rather at the cell signal transduction level. Therefore, BayK might play a more specific role in the reprogramming process. When 129 MEFs were transduced with empty retrovirus (negative control); no colonies observed. When 129 MEFs were transduced with OK without small molecules; few small flattened colonies with weak ALP expression present. ESC-like iPSC colonies were observed 14-21 days after 129 MEFs were transduced with OK and treated with BIX/BayK; these ESC-like colonies exhibited strong ALP expression. When OK-transduced MEFs were treated with BIX in combination with BayK, a significant increase in the number of ALP^+ colonies that closely resemble the mESC morphology could be observed (~7 colonies) as compared to OK-transduced MEFs treated with BIX alone (~2 colonies). Further characterization of these primary iPSC colonies showed that they were positive for typical

pluripotency markers such as Oct4, Sox2, Nanog, and SSEA1 as determined by immunofluorescence.

iPSCs Obtained from MEFs Transduced with OK and Treated with BIX/BayK have Pluripotency Properties Characteristic of mESCs.

To further confirm and characterize that OK transduction and BIX/BayK treatment can induce MEFs to become iPSCs, we used primary MEFs derived from $OG2^{+/-}$/$ROSA26^{+/-}$ (OG2) transgenic mice, which contain an Oct4-GFP reporter (Do, J. T. and Scholer, H. R., *Stem Cells,* 22:941-949 (2004)). Once reprogrammed, these cells could then be used to conveniently assess chimera and germline competency. Similarly to 129 MEFs, OG2 MEFs transduced with OK could generate iPSCs when treated with a combination of BayK/BIX (OK2B iPSCs) (FIG. **3**). GFP^+ iPSC colonies could be first detected on day 14-21 after viral transduction and compound treatment. When OG2 MEFs were transduced with OK and not treated with any compounds, only a few small colonies appeared for an average of 0.5±0.7 colony per 3.5×10^4 cells. These colonies were difficult to passage and therefore were not studied any further. Treatment of OK transduced OG2 MEFs with BIX alone readily and reproducibly enabled reprogramming as compared to OK alone, with 2.5±0.7 colonies per 3.5×10^4 cells. There was a further significant improvement in the reprogramming efficiency when OG2 MEFs transduced with OK were treated with the combination of BIX (2 μM) and BayK (2 where we observed 7.7±1.5 colonies per 3.5×10^4 cells (FIG. **3**). Treatment of OK-transduced OG2 MEFs with BayK alone, in the absence of BIX, did not increase reprogramming efficiency above OK-transduced MEF control.

OK2B colonies were picked out and serially expanded on irradiated MEF feeder cells in the conventional mESC growth conditions in the absence of small molecules for more than 20 passages. Staining and/or RT-PCR (FIG. **4**A) showed that these GFP^+ OK2B iPSCs express typical pluripotency markers, including ALP, Nanog, Sox2, Oct4, SSEA1, c-Myc, eRas, Esg1, Ecat1, and Fgf4. RT-PCR assay also demonstrated that OK2B iPSCs expressed endogenous Oct4 and Klf4 (FIG. **4**A). Bisulphite genomic sequencing analyses of the Nanog promoter revealed that it is demethylated in OK2B iPSCs similarly to the mESC control (R1), while the MEFs' Nanog promoter was hypermethylated (FIG. **4**B). This result further suggests a reactivation of the stem cell transcription program in these OK2B iPSCs. In addition, transcriptome analysis showed that expression profile of OK2B iPSCs is greatly similar to the one of mESCs with a Pearson correlation value of 0.96, while significantly different to MEFs' profile with a Pearson correlation value of 0.84 as exemplified in the clustering analysis.

For comparison of OK2B transcriptome with mES cells and MEF cells, transcriptome analysis was carried out. RNA was extracted from OK2B iPS cells at passage 13 using Qiagen RNAeasy Mini Kit. RNA expression data for OK2B iPSCs was generated from polyA RNA using GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix). Expression data for MEF cells and mES cells were obtained from the Gene Expression Omnibus (GEO) website www.ncbi.nlm-.nih.gov/geo/. mES cells data registry number: GSM198062. GSM198063, and GSM198064. MEF cell data registry number: GSM198070 and GSM198072. Pre-processing, normalization (GC-RMA) and hierarchical clustering were performed using dChip (biosun1.harvard.edu/complab/dchip/; (Distance metric:correlation (Pearson); linkage method: centroid; gene ordering: by cluster tightness). p value for OK2B iPSC versus MEF cells: 0.84; OK2B iPSC versus mES cells: 0.96. p values were obtained using a Pearson correlation test.

OK2B iPSCs Differentiate into Cells from all Three Germ Layers and Contribute to Germline Transmission.

OK2B iPSCs could efficiently form embryoid bodies (EB) in suspension, which could differentiate into endodermal cells (Albumin and Pdx1), mesodermal cells/cardiac muscle cells (CT3) and ectodermal cells/neurons (βIII-tubulin, Tuj1), derivatives of the three primary germ layers. In addition, OK2B iPSCs could efficiently incorporate into the inner cell mass of a blastocysts following aggregation with an 8-cell embryo, and lead to chimerism with germline contribution in vivo after the aggregated embryos were transplanted into pseudo-pregnant mice. Moreover, mating of one adult male progeny obtained from these blastocysts with a female CD1 wild-type mouse led to the production of $LacZ^+$ progeny, three of which showed Oct4-GFP^+ gonads further validating that these iPSCs could contribute to germline transmission. These in vitro and in vivo characterizations confirm retroviral transduction with only two genes, OK, and in conjunction with BIX/BayK treatment are sufficient to reprogram MEFs into iPSCs, which are phenotypically and functionally similar to the classic mESCs.

Discussion

The studies presented here provide a proof-of-principle demonstration that small molecules can be identified from rationally designed phenotypic screens to functionally replace viral transduction of certain TF(s) as well as improve reprogramming efficiency in generating iPSCs from a general cell-type, like MEFs. Such a chemical approach for the generation of iPSCs, which offers more precise and temporal control of the target/process, would be advantageous over the genetic manipulation with oncogenes that may also introduce harmful hard-to-detect insertional genomic alterations. Similar strategies are being used to find additional small molecules that may ultimately allow reprogramming of lineage-restricted cells to pluripotent or multipotent state in a completely chemically defined condition. BIX was originally identified and characterized as a specific inhibitor for G9a HMTase (Kubicek, S. et al., *Mol Cell,* 25:473-481 (2007)). It has been shown to reduce H3K9me2 levels at G9a target genes (Feldman, N. et al., *Nat Cell Biol,* 8:188-194 (2006)). Interestingly, histone H3K9 methylation, mediated by G9a, and heterochromatinization represent a highly specific mechanism for epigenetic silencing of embryonic genes such as Oct4 and Rex1 (Feldman, N. et al., *Nat Cell Biol,* 8:188-194 (2006)). Furthermore, it was also demonstrated that knock-down of G9a can assist in fusion-based reprogramming of adult neuronal cells (Ma, D. K. et al., *Stem Cells,* 26:2131-2141 (2008)). It is therefore fitting that we previously observed that BIX can facilitate the generation of iPSCs from NPCs transduced with either OK or Klf4/Sox2/c-Myc (Shi, Y. et al., *Cell Stem Cell,* 2:525-528 (2008)), suggesting that it can compensate for the exogenous expression of Sox2 or Oct4. However, NPCs already express significant levels of Sox2, which might cause these cells to be more susceptible to reprogramming in the conditions mentioned above. This present study aimed at identifying small molecules that can enable reprogramming of MEFs, which do not express any of the TFs deemed necessary for reprogramming. It was fortuitous that we identified BIX in both the NPC and MEF screens, and further confirm this molecule has a role in enabling and improving the generation of iPSCs from somatic cells. Given BIX's characterized mechanism of action, our studies potentially identified a molecular target whose loss-of-function via pharmacologi-

cal inhibition is sufficient to compensate for the gain-of-function of an essential iPSC reprogramming gene. It further mechanistically links a specific epigenetic process, inhibition of G9a-mediated H3K9me2, to iPSC generation. BIX may function to facilitate shifting of the epigenetic balance from a silenced state of pluripotency genes to an active transcription state. Obviously, combination of BIX with other chromatin-modifying small molecules, which have different targets and mechanisms of action, such as RG108 could be exploited for better reprogramming. On the other hand, our observation that BayK, with a characterized activity as a specific L-type calcium channel agonist (Schramm, M. et al., *Nature,* 303:535-537 (1983)), improves reprogramming efficiency is intriguing. L-type calcium channels are known to mediate intracellular processes in different tissues such as blood pressure regulation, smooth muscle contractility, insulin secretion, cardiac development, etc (Tosti, E., *Reprod Biol Endocrinol,* 4:26 (2006)). Furthermore, activation of L-type calcium channels by different agonists, including BayK, has been shown to induce intracellular signaling through CREB activation, sarcoplasmic reticulum Ca^{2+} release, and change in cAMP activity. More importantly, some reports suggest that calcium might play a role in the control of mES cell proliferation (Heo, J. S. et al., *Am J Physiol Cell Physiol,* 290:C123-133 (2006)). However, in our hands, treatment of mES cell with 2 μM BayK alone or in combination with 1 μM BIX does not lead to a change in proliferation (FIG. **5**). Furthermore, treatment of OG2 MEF with 2 BayK alone or in combination with 1 μM BIX does not induce SOX2 expression (FIG. **6**). Needless to say, more work needs to be performed to dissect the precise mechanism by which BayK impacts the reprogramming process. However, it is interesting to find that a small molecule with activity in signaling pathways that have not been previously linked to reprogramming can significantly enhance its efficiency. So far, it is the first small molecule of its type, aside from Wnt3 protein (Marson, A. et al., *Cell Stem Cell,* 3:132-135 (2008)), to show an effect on reprogramming without acting directly on chromatin modifiers. As, up to date, most of the other small molecules found to impact reprogramming appear to directly modify the epigenetic status of the cell: i.e., BIX (Shi, Y. et al., *Cell Stem Cell,* 2:525-528 (2008)), valproic acid (Huangfu, D. et al., *Nat Biotechnol,* 26:795-797 (2008)) and 5'azacytidine (Mikkelsen, T. S. et al., *Nature,* 454:49-55 (2008)). Importantly, BayK seems to have several important characteristics that would be ultimately desirable for a molecule to be therapeutically relevant for in vivo reprogramming and/or regeneration. The fact that it does not act/reprogram on its own, but needs the presence of BIX to exert its effects suggests that cells that are already undergoing a form of reprogramming, perhaps caused by injury, might be more susceptible to its effect. This might allow us to ultimately reprogram the target cell in a more specific manner, without impacting healthy cells systemically, as direct epigenetic modifiers might.

In summary, we have identified defined small molecule conditions, i.e., BIX, and combinations of BIX/BayK, or BIX/RG108, which can enable and improve reprogramming of fibroblasts into iPSCs in conjunction with the transduction of only two TFs: Oct4 and Klf4. This study further confirms the usefulness of a phenotypic screening approach in identifying small molecules that can effectively compensate for the viral transduction of an essential iPSC TF, such as Sox2 in this study or Oct4 as previously reported (Shi, Y. et al., *Cell Stem Cell,* 2:525-528 (2008)). Ultimately, phenotypic small molecule screens may lead to the identification of small molecule that will become powerful tools in providing us with new insights into the reprogramming process, and may ultimately be useful to in vivo stem cell biology and therapy.

Experimental Procedures

MEFs Derivation

129S2/SvPasCrlf or $ROSA26^{+/-}/OG2^{+/-}$ MEFs were derived according to the protocol reported on WiCell Research Institute website: "Introduction to human embryonic stem cell culture methods." Animal experiments were performed according to the Animal Protection Guidelines of the Max Planck Institute for Biomolecular Research, Germany.

Retrovirus Transduction and Compounds

pMX-based retroviral vectors for mouse Oct4, Klf4, c-Myc and Sox2 were obtained from Addgene (Cambridge, Mass.). The viral production and transduction process was performed as described (Takahashi, K. et al., *Cell,* 131:861-872 (2007)). The synthesis and full characterization of compound BIX-01294 was as previously described (Kubicek, S. et al., *Mol Cell,* 25:473-481 (2007)) and Bayk8644 was purchased from EMD/Calbiochem Biochemical (San Diego, Calif.).

Screen for iPSC Generation from MEFs

For the primary and secondary screens, a collection of known compounds was used. This collection was composed of roughly 2000 known bioactive molecules that are commercially available, including FDA-approved drugs, known inhibitors and activators of characterized enzymes (including LOPAC collection from Sigma-Aldrich (St. Louis, Mo.), Known Bioactive Library from BIOMOL (Plymouth Meeting, Pa.) and non-overlapping known compounds from EMD Calbiochem (San Diego, Calif.)).

Primary 12952/SvPasCrlf (primary screen) or $ROSA26^{+/-}/OG2^{+/-}$ (secondary screen) MEFs were plated onto Matrigel (1:50; BD Biosciences, Bedford, Mass.) coated dishes at a density of 3.5×10^4 cells per well of a 6-well plate. Twenty-four hours later, these cells were transduced overnight with defined retroviruses at 37° C., 5% CO_2. Twelve to fourteen hours later, the media on the transduced cells was changed to mESC medium [Knockout DMEM, 10% ES-qualified FBS, 10% Knockout serum replacement, 1% Glutamax, 1% Non-essential amino acids, penicillin/streptomycin, 0.1 mM β-mercaptoethanol, 1% EmbryoMax ESC Qualified Nucleosides (Millipore, Temecula, Calif.), and 10^3 U/ml LIF (Millipore)] (all products were from Invitrogen, Carlsbad, Calif., except where mentioned). On that same day, individual small molecules from our known drug collection were added to the cells at a range between 0.5 and 2 μM. Compound treatment was continued for 10-14 days; the cells were fixed and stained on day 14-21 using a standard ALP detection kit (Millipore). For the second screen, 1 μM BIX was added to the mESC medium 1 day after transduction. Five days later, in addition to 1 μM BIX, individual small molecule from the known drug collection was added to each well, at a range between 0.5 and 2 μM. Mouse ESC media with defined small molecules was refreshed every three days until colonies with a similar morphology to mESCs were observed, which was usually between 14-21 days after transduction. In addition to the confirmed compounds as indicated in the text, primary hits from the second synergist screen that were not further followed up also include PD173074, reversine, 5' azacytidine, pluripotin, and dexamethasone. Further characterization studies and repeats were carried either on primary 129S2/SvPasCrlf or $ROSA26^{+/-}/OG2^{+/-}$ MEFs. When $ROSA26^{+/-}/OG2^{+/-}$ MEFs were used, the iPSC colonies

could also be identified through GFP expression, as a marker of Oct4 expression. Once iPSC colonies were identified, they were picked for expansion on MEF feeder cells in mESC medium. Some colonies were expanded in the presence of the MEK inhibitor, PD0325901 at concentration of 0.5-2 μM to further confirm their pluripotentiality.

Immunocytochemistry and Immunofluorescence Assay

ALP staining was performed according to manufacturer's instruction using the Alkaline Phosphatase Detection Kit (Millipore). For immunofluorescence assay, cells were fixed in 4% paraformaldehyde 15 minutes at room temperature (RT) and washed with PBS. They were then incubated in blocking buffer (BB) [0.3% Triton X-100 (Sigma-Aldrich), 10% normal donkey serum (Jackson ImmunoResearch Laboratories Inc) in PBS (Invitrogen)] 30 min at RT. They were then incubated with primary antibody overnight at 4° C. in BB. Afterward, cells were washed with PBS and incubated with secondary antibody in BB for 45-60 min at RT. Primary antibodies were; mouse anti-Oct4 (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), mouse anti-SSEA1 (1:200) (Santa Cruz Biotechnology Inc.), rabbit anti-Nanog (1:500) (Abcam Inc., Cambridge, Mass.), mouse anti-Sox2 (1:200) (Millipore), rabbit anti-Pdx1 (1:200) (a kind gift from Dr. C. Wright), mouse anti-βIII-Tubulin (Tuj1) (1:500) (Covance Research Products Inc., Denver, Pa.), mouse anti-cardiac troponin T (CT3) (1:200) (Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, Iowa), rabbit anti-albumin (DAKO). Secondary antibodies were Alexa Fluor555 donkey anti-mouse or rabbit IgG (1:500) (Invitrogen). Nuclei were detected by DAPI (Sigma-Aldrich) staining. Images were captured using a Nikon Eclipse TE2000-U/X-cite 120 EXFO microscope with a photometric CoolSnap HQ2 camera.

RT-PCR Assay

RNA was extracted from iPSCs and control cell lines using the RNeasy Plus Mini Kit in combination with QIAshredder. The RNA was converted to cDNA using iScriptTMcDNA Synthesis Kit (BioRad, Hercules, Calif.). Amplification of specific genes was done using primers previously published (Takahashi, K. et al., *Cell,* 131:861-872 (2007); Takahashi, K. and Yamanaka, S., *Cell,* 126:663-676 (2006)) and Platinum PCR SuperMix (Invitrogen) on a Mastercycler ep gradient PCR machine (Eppendorf).

Methylation Assay

DNA from R1, OG2 MEFs and OK iPSCs (passage 10) cells was isolated using the Non Organic DNA Isolation Kit (Millipore). The DNA was then treated for bisulfite sequencing with the EZ DNA Methylation-Gold Kit™ (Zymo Research Corp., Orange, Calif.). The treated DNA was then used to amplify sequences of interest. Primers used for promoter fragment amplification were as previously published (Blelloch, R. et al., *Stem Cells,* 24:2007-2013 (2006)). The resulting fragments were cloned using the TOPO TA cloning Kit for sequencing (Invitrogen) and sequencing was done.

Aggregation of iPSCs with Zona-Free Embryos

iPSCs were aggregated with denuded post-compacted eight-cell stage embryos to obtain aggregate chimeras. Eight-cell embryos (B6C3F1) were flushed from females at 2.5 dpc and cultured in microdrops of KSOM medium (10% FCS) under mineral oil. Clumps of iPSCs (10-20 cells) after short treatment of trypsin were chosen and transferred into microdrops containing zona-free eight-cell embryos. Eight-cell embryos aggregated with iPSCs were cultured overnight at 37° C., 5% CO_2. Aggregated blastocysts that developed from eight-cell stage were transferred into one uterine horn of a 2.5 dpc pseudopregnant recipient. One adult male chimaera was mated with a female CD1 wild-type mouse. X-gal staining showed that six F1 embryos obtained from this natural mating of chimeric mouse and wild-type mouse were generated by germline transmission.

Statistical Analysis

Bar graphs and statistical analyses were performed using a standard t-test on the Excel.

Microarray Analysis

OK2B iPSCs were grown in complete mES cell media on gelatin (Millipore, Temecula, Calif.) for 2 days [Knockout DMEM, 10% ES-qualified FBS, 10% Knockout serum replacement, 1% Glutamax, 1% Non-essential amino acids, penicillin/streptomycin, 0.1 mM β-mercaptoethanol, 1% EmbryoMax ESC Qualified Nucleosides (Millipore), and 10^3 U/ml LIF (Millipore)] (all products were from Invitrogen, Carlsbad, Calif., except where mentioned). RNA from duplicate wells was then isolated using RNAeasy Mini Kit (Qiagen, Valencia, Calif.). Total RNA samples were amplified and labeled using the MessageAmp II-Biotin Enhanced Kit (Ambion, Austin, Tex.). The amplified labeled samples were then hybridized to Mouse Genome 430 2.0 Arrays (Affymetrix) and analysis was performed using hierarchical clustering (Pearson, log-transformed, row-centered values) using GenePattern (world wide web at: broad.mit.edu/cancer/software/).

Proliferation Assay

mES R1 cells were plated onto gelatin-coated 6-well plates at a density of 2×10^5 cells/well in complete mES cell media. Upon cell attachment, app. 12 hours, the cells were treated either with DMSO, 1 μM BIX, 2 μM BayK, and a combination of both, in triplicate. At 15, 24 and 48 hours, the cells were detached using trypsin, and counted using a hemocytometer. Trypan blue (Sigma-Aldrich, St. Louis, Mo.) was used for dead cell exclusion.

Assessment of SOX2 Expression after Compound Treatment

$OG2^{+/-}/ROSA26^{+/-}$ MEFs were plated onto 6-well plate at a density of 3.4×10^4 cells per well. On the following day, the cells were treated with DMSO, 1 μM BIX, 2 μM BayK, and a combination of both, in triplicate, for 6 days. The media was refreshed at day 3. RNA from each well was then isolated using the RNAeasy Mini Kit (Quiagen). Reverse transcription of the RNA was performed using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, Calif.). Amplification of endogenous Sox2 was done using primers previously published (Takahashi, K., Okita, K., Nakagawa, M., and Yamanaka, S. (2007). Induction of pluripotent stem cells from fibroblast cultures. Nat Protoc 2, 3081-3089; Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-676) with Platinum PCR SuperMix (Invitrogen) on a Mastercycler® ep gradient PCR machine (Eppendorf).

Example 3

This example demonstrates that incubation of mammalian cells with transcription factor proteins is sufficient to induce pluripotency.

Gene Construction:

In order to obtain the high level protein expression in *E. coli*, all four human TF gene codon region were optimized first (G A Gutman and G W Hatfield (1989). PNAS. vol. 86. pp: 3699-3703), and full-length synthesized using DNA oligo based/PCR gene assembling technology (Danilo R Casimiro, Peter E Wright & H Jane Dyson. (1997). Structure. Vol. 5. pp: 1407-1412.). Poly-arginine tag:

ESGGGGSPGRRRRRRRRRRR (SEQ ID NO:2) was added to each protein C-terminal in design (Gump J M, Dowdy S F. (2007) Trends Mol Med. 2007 October; 13(10):443-8). The final DNA fragment was flanked with NdeI and XhoI site, and inserted into pET41 expression vector NdeI-XhoI sites for protein expression. Each plasmid were verified with DNA sequence, then transformed into BL21start competent cells for recombinant protein production using auto-induction medium overnight (Studier F W, (2005) Protein Expr Purif. 41 (1). Pp: 207-234.).

Protein Preparation

Escherichia coli BL21(DE3) cells were transformed with pET-Oct4-PTD ("PTD" refers to protein transduction domain), pET-Sox2-PTD, pET-Klf4-PTD, and pET-c-Myc-PTD separately, and the protein expression was done using the auto-induction method (Studier F. W., Protein Expression and Purification, 41 (2005) 207-234.). Inclusion bodies were solubilized and the proteins were refolded as described (LaFevre B M, Wu S. & Lin X. Molecular Cancer Therapeutics 7, 1420-1429, Jun. 1, 2008. doi: 10.1158/1535-7163; Medynski D., Tuan M., Liu, W., Wu, S. & Lin, X. Protein Expression and Purification Vol. 52, 395-402, April 2007; Hou W., Medynski D., Wu, S., Lin, X. & Li, L Y. Clinical Cancer Research Vol. 11, 5595-5602, Aug. 1, 2005).

Briefly, *E. coli* containing an expression plasmid was inoculated into 1.0 L liter of Luria-Bertani Broth containing kanamycin, induced with 500 umol/L IPTG at A600 nm=0.6, and agitated for 3 hours at 37 C. The cells were collected by centrifugation, and the pellet subjected to freeze- and thaw cycles. The inclusion bodies released were washed extensive with a buffer containing 20 mmol/L tris, 100 mmol/L NaCl, 1% TritonX-100 (pH8.0) and dissolved in a buffer containing 8 mol/L urea, 0.1 mol/L Tris, 1 mmol/L glycine, 1 mmol/L EDTA, 10 mmol/L b-mercaptoethanol, 10 mmol/L DTT, 1 mmol/L reduced glutathione, 0.1 mmol/L oxidized glutathione (pH 10) with a A280 nm=2.0. The solubilized inclusion bodies were refolded with a rapid dilution method as described (Lin X L, Lin Y Z, Tang J., Methods Enzymol 1994. 241. 195-224; Lin X, Koelsh G., Wu. S, Downs D, Dashti A. Tang J. Proc Natl Acad Sci USA. 2000; 97. 1556-1560; Kim Y T. Downs D. Wu S, et al. Eur J Biochem 2002. 269: 5669-77; Michelle LaFevre-Bernt, Shili Wu, and Xinli Lin. (2008). Molecular Cancer Therapeutics. 7: pp: 1420-1429). The refolded protein was concentrated by N2-ultrafiltration and purified by size exclusion chromatography using Sephacryl S 300. The endotoxin concentration in each of protein preparation was less than 100EU/mg. Most refold protein samples have solubility at least >1.5 mg/ml.

Refolded proteins were concentrated using tangential flow filtration, purified using size exclusion chromatography with a Superdex-200 column (XK26×850-mm, GE, Piscataway, N.J.), and confirmed using SDS-PAGE.

Mouse fibroblasts were grown in mESC medium supplemented with 8 μg/ml of either Oct4/Sox2/Klf4 or Oct4/Sox2/Klf4/Myc (all proteins comprising poly-Arg as described above) for 6-8 hours, washed, and incubated for 2-3 days in mESC media without the above-listed transcription factors. This (4-12 hours with, 1-3 days without) was repeated a number (1, 2, 3, 4, or more) of times and then the cells were cultured in mESC for two weeks. At the end of this period, the cultures were determined to contain pluripotent cells by colony morphology and marker expression. Notably, it was found that constant incubation of the cells with the transcription factors (i.e., without the 1-3 day period without the proteins)) was toxic to the cells. While it was not necessary, the cells were sometimes incubated with MEK inhibitor (PD0325901) and/or GSK inhibitor (CHIR99021) and/or TGFbeta inhibitor (SB431542) and the presence of these agents improved efficiency and speed of development of pluripotent cells.

We tested the TGFβ receptor inhibitor (A-83-01), MEK inhibitor (PD0325901) and GSK-3 inhibitor (CHIR99021), on mouse fibroblast cells that were treated with exogenous Oct4, Klf4, Sox2 and c-Myc polypeptides, for their effects on reprogramming kinetics and efficiency.

Mouse fibroblasts were grown in mESC growth medium [Knockout DMEM (Invitrogen) supplemented with 10% ES-FBS+10% KSR, 0.1 mM 2-ME, 1% Glutamax, 1% Non-essential amino acids, 1% EmbryoMax ESC Qualified Nucleosides (Millipore), and 10^3 units/ml LIF (ESGRO, Chemicon International)] supplemented with 8 μg/ml of Oct4/Sox2/Klf4/Myc (all four transcription factors are tagged with poly-Arg ESGGGGSPGRRRRRRRRRRR; SEQ ID NO:2) for 4-12 hours, followed by culturing the cells in the absence of the Oct4/Sox2/Klf4/Myc polypeptides for one day. This procedure was repeated 4 times. The cells were then cultured in mESC medium for another two weeks. iPSC clones were detected by colony morphology and expression of pluripotency markers. An intermediate iPSC clone, which expresses ALP but not Oct4 (Oct4), was dissociated with Trypsin-EDTA. The clone was then expanded by continuous culturing with regular mESC medium or medium supplemented with 0.5 μM PD0325901, 3 μM CHIR99021, 0.5 μM A-83-01. The number of Oct4-positive colonies were counted 2 weeks after small molecule treatment (FIG. **14**). The treatment of PD0325901, CHIR99021, and A-83-01 increases reprogramming efficiency and accelerates reprogramming kinetics by promoting intermediate piPS cells to stable and fully reprogrammed piPS cells.

Example 4. Reprogramming of MEF

We tested the effects of a GSK-3 inhibitor, a TGFβ receptor inhibitor, and a MEK inhibitor on MEFs that were retrovirally transduced with either (1) Oct4, Klf4, Sox2 and c-Myc; or (2) Oct4, Klf4, and Sox2, on reprogramming kinetics and efficiency.

Methods:

MEFs were derived from OG2 mice according to the protocol reported on WiCell Research Institute website: "Introduction to human embryonic stem cell culture methods". OG2 transgenic mice carry both GFP under the control of the Oct4 promoter. MEFs were transduced with either (1) Oct4, Klf4, Sox2 and c-Myc; or (2) Oct4, Klf4, and Sox2 as described (Takahashi & Yamanaka, Cell. 2006; 126:663-676). Twenty four hours later, transduced MEFs were seeded in 6-well plate at 1×10^5 cells/well and incubated with mESC growth medium: Knockout™ DMEM, 7% ES Cell-Qualified fetal bovine serum, 10% Knockout Serum Replacement, 1% Glutamax, 1% Non-essential amino acids, 1% penicillin/streptomycin, 0.1 mM β-mercaptoethanol and 10^3 U/ml mLIF. Transduced MEFs were then treated with chemicals or chemical combinations, including GSK-3 inhibitor CHIR99021 (5 μM), TGFβ receptor inhibitor SB431542 (2 μM), MEK inhibitor PD0325901 (0.1 μM) for 11 days (for Oct4, Klf4, Sox2 and c-Myc transduced MEFs) or 13 days (for Oct4, Klf4 and Sox2 transduced MEFs), and EGFP positive colonies were counted.

Results:

For Oct4, Klf4, Sox2 and c-Myc transduced MEFs, we tested the effect of TGFβ receptor inhibitor (SB431542), alone or in combination with GSK-3 inhibitor (CHIR99021)

and/or MEK inhibitor (PD0325901), on reprogramming efficiency (11-day treatment). We found that treatment with 2 μM SB431542 results in a ~1.8% reprogramming efficiency, treatment with the combination of 2 μM SB431542 and 5 μM CHIR99021 results in a ~2.2% reprogramming efficiency, and that treatment with the combination of 2 μM SB431542, 0.1 μM PD0325901 and 5 μM CHIR99021 results in a ~0.4% reprogramming efficiency. Reprogramming efficiency for Oct4, Klf4, Sox2 and c-Myc transduced MEFs untreated with TGFβ receptor inhibitor, GSK-3 inhibitor or MEK inhibitor is ~0.2%.

For Oct4, Klf4, and Sox2 transduced MEFs, we also tested the effect of TGFβ receptor inhibitor (SB431542), alone or in combination with GSK-3 inhibitor (CHIR99021) and/or MEK inhibitor (PD0325901), on reprogramming efficiency (13-day treatment). We found that treatment with 2 μM SB431542 results in a ~1.2% reprogramming efficiency, treatment with the combination of 2 μM SB431542 and 5 μM CHIR99021 results in a ~1.7% reprogramming efficiency, and that treatment with the combination of 2 μM SB431542, 0.1 μM PD0325901 and 5 μM CHIR99021 results in a ~0.8% reprogramming efficiency. Reprogramming efficiency for Oct4, Klf4, and Sox2 transduced MEFs untreated with TGFβ receptor inhibitor, GSK-3 inhibitor or MEK inhibitor is ~0.1%.

Example 5. Reprogramming of Human Primary Fibroblast

We tested known inhibitors of the TGFβ receptor and MEK on 1×10^4 [Feng, B. et al., *Cell Stem Cell* 4, 301-12 (2009)] human primary fibroblasts (CRL2097 or BJ) that were retrovirally transduced with the 4TFs (Oct4, Klf4, Sox2 and c-Myc), for their effects on reprogramming kinetics and efficiency. On day 7 (D7) post-infection, the compounds were added, individually or in combinations, and the cultures were examined for iPSCs over the next 1-3 weeks.

On day 7 post-treatment (D14), we observed the strongest effect in the cultures treated with a combination of ALK5 inhibitor SB431542 (2 μM) and MEK inhibitor PD0325901 (0.5 which resulted in ~45 large ALP^+ colonies with characteristic hESC-like morphology, of which over 24 colonies were TRA-1-81$^+$, and about 6-10 colonies stained positive for SSEA4 and NANOG, a mature pluripotency factor that is not ectopically introduced. Moreover, the treated cultures showed high level expression of endogenous mRNA for the pluripotency genes. In contrast, no $NANOG^+$ colonies were observed in the untreated control cultures (FIG. **7***a*) or in cultures that were treated with PD0325901 alone (FIG. **7***a*). However, in the cultures treated with only SB431542, we still observed 1-2 ALP^+ hESC-like colonies (FIG. **7***a*). Importantly, the combined effect of both the inhibitors (FIGS. **7***b* & **7***c*), as well as the individual effect of SB431542 was dose dependent.

When we maintained the SB431542 plus PD0325901 treated cultures for 30 days without splitting, we obtained about 135 iPSC colonies per well, a >100 fold improvement in efficiency over the conventional method. Consistent with previous reports [Takahashi, K. et al., *Cell* 131, 861-72 (2007)], in untreated controls carrying 4TFs, we observed 1-2 iPSC colonies in addition to several granulate colonies. These granulate structures have been suggested to be partially reprogrammed colonies [Takahashi, K. et al., *Cell* 131, 861-72 (2007)]. We also observed granulate colonies in the SB431542 treated cultures, which outnumbered by several fold the few hESC-like colonies. Interestingly, the number of granulate colonies was dramatically reduced in the combined SB431542 and PD0325901 treatment, which resulted in a concomitant increase in the number of hESC-like colonies. This suggested that a combined inhibition of ALK5 and MEK may guide partially reprogrammed colonies to a fully reprogrammed state and thereby improve the overall reprogramming process. Moreover, the fact that we observed improved induction of iPSCs as early as 7 days post-treatment suggests that treatment with these small molecules not only improved the efficiency of the reprogramming process but may also have accelerated its kinetics.

Two compound treatment also resulted in a larger number of alkaline phosphatase-positive colonies compared to untreated controls when the reprogramming factors were introduced using a lentiviral, rather than a retroviral system.

The demonstration that TGFβ and MEK-ERK pathway inhibition improved fibroblast reprogramming suggested critical roles for these two signaling pathways and MET mechanisms in the process. Consistently, addition of TGFβ had an inhibitory effect on 4 factor-mediated reprogramming of fibroblasts. TGFβ and its family members play important contextual roles in self-renewal and differentiation of ESCs [Watabe, T. and Miyazono, K., *Cell Res.* 19, 103-15 (2009)]. Moreover, TGFβ is a prototypical cytokine for induction of epithelial mesenchymal transition (EMT) and maintenance of the mesenchymal state [Willis, B. C. and Borok, Z., *Am. J. Physiol. Lung Cell Mol. Physiol.* 293, L525-34 (2007)]. A major end point of this signaling, in this context, is down regulation of E-cadherin [Thiery, J. P. and Sleeman, J. P., *Nat. Rev. Mol. Cell Biol.,* 7, 131-42 (2006)]. E-cadherin has been shown to be important for the maintenance of pluripotency of ESCs and has been recently suggested to be a regulator of NANOG expression [Chou, Y. F. et al., *Cell* 135, 449-61 (2008)]. Therefore inhibition of TGFβ signaling, which results in de-repression of epithelial fate, could benefit the reprogramming process in multiple ways. ERK signaling also promotes EMT [Thiery, J. P. and Sleeman, J. P., *Nat. Rev. Mol. Cell Biol.* 7, 131-42 (2006)], and is downstream of TGFβ in the process [Chou, Y. F. et al., *Cell* 135, 449-61 (2008)]. We had previously shown that the effect of reversine, a small molecule which can reprogram myoblasts to a multipotent state, is mediated in part through inhibition of MEK-ERK [Chen, S. et al., *Proc. Natl. Acad. Sci. USA* 104, 10482-87 (2007)]. This may explain the effect observed in reprogramming when it was combined with TGFβ inhibition.

Methods

Cell Culture

Primary skin fibroblasts CRL2097 and BJ (neonatal foreskin) were purchased from ATCC. All cell culture media reagents were purchased from Invitrogen Corporation, CA. The cells were maintained in DMEM (10313-021) containing 10% FBS (10439-024), 1×MEM Non-Essential Amino acid (11140-050), 1× Glutamax (35050-061), 10 mM Hepes (15630-080) and 0.11 mM 2-Mercaptoethanol (21985-023). Cells were passaged 1:5 using 0.05% (1×) trypsin-EDTA (25300-054).

Plasmids

The pMXs vector encoding the human cDNAs for OCT4, SOX2, c-MYC and KLF4, described before [Takahashi, K. et al., *Cell* 131, 861-72 (2007)], were obtained from ADDGENE. Mouse Slc7a1 ORF was cloned into pWPXLD (Addgene), as described previously [Takahashi, K. et al., *Cell* 131, 861-72 (2007)].

Retroviral Infection and iPS Cell Generation

Lentiviruses carrying OCT4, NANOG, SOX2 & LIN28 were produced as described before [Yu, J. et al., *Science* 318,

1917-20 (2007)]. For retrovirus production, PLAT-E packaging cells were plated at 1×10^6 cells/well of a 6-well plate. After 24 hours, the cells were transfected with pMXs vectors carrying OCT4, SOX2, c-MYC and KLF4 cDNAs using Fugene 6 transfection reagent (Roche) according to manufacturer's instructions. Twenty-four hours after transfection, the medium was replaced with fresh medium and the plate was transferred to 32° C. for retrovirus production. The viruses were collected at 48 hours and 72 hours, and filtered with 0.45 μm filter before transduction.

The Slc7a1-expressing human fibroblast cells were seeded at 1×10^5 cells/well of a 6 well plate on the day 1. On day 2, 0.25 ml of each retroviral supernatant was added to the cells in the presence of 6 μg/ml polybrene. A second round of transduction was done on day 3. Infection efficiency was estimated by fluorescence microscopy on cells transduced in parallel with GFP or RFP gene-carrying retroviruses. Seven days after initial transduction, fibroblasts were harvested by trypsinization and re-plated at 1×10^4 cells/well of a 6 well plate coated with matrigel (1:50 dilution, cat 354234, BD Biosciences). For compound treatment, the cells were cultured in human reprogramming medium (DMEM/F12, 20% Knockout serum replacer, lx MEM Non-Essential amino acid, lx glutamax, 0.11 mM 2-Mercaptoethanol, 20 ng/ml bFGF and 1,000 U/ml LIF) and were treated with 2 μM SB431542 (Stemgent), 0.5 μM PD0325901 (Stemgent), or combinations of the compounds. The media were changed every 2-3 days depending on the cell density. Seven days after compound treatment, either the plates were fixed and stained for Alkaline phosphatase (ALP) activity, or stained for protein markers, or the cultures were continued with or without indicated splitting by trypsinization till day 30. For split cultures, the cells were split (1:4) and re-plated onto irradiated CF-1 MEF feeder layer (2.5×105 cells/well) in each well of 6 well plate and were split (1:10) again on day 21. The cells were maintained in the same media and compound cocktail described above except for the concentrations of PD0325901 (0.5 μM for D14 and 1 μM for D21) and SB431542 (0.5-1 μM after D14). The iPSC colonies were subsequently maintained in conventional hESC media in the absence of the above compounds.

Alkaline Phosphatase Staining and Immunocytochemistry

Alkaline phosphatase staining was performed using ALP detection kit (cat no: SCR004, Chemicon) according to the product instructions. For immunocytochemistry, cells were fixed in 4% paraformaldehyde (10 min, RT), washed twice with PBS, blocked using 5% normal donkey serum (Chemicon) and 0.1% TritonX-100 (15 min, RT) and then treated with primary antibodies overnight at 4° C. The primary antibodies used were anti-NANOG (cat no: AB9220, Chemicon, 1:1,000); anti-OCT4 (cat no: sc-5279, Santa Cruz biotech, 1:200), anti-SSEA 4 (cat no: mab4304, Chemicon, 1:500), anti-Tra-1-81 (cat 560123, BD Biosciences, 1:100), anti-Tra-1-81 (mAb 4381, Chemicon, 1:500), anti-βIII TUBULIN (cat no: MMS-435P, Covance Research Products Inc, 1:1000), anti-PDX 1 (1:500) (a kind gift from Dr. C. Wright), anti-BRACHYURY (cat No: AF2085, R & D, final concentration 0.2 μg/ml). The cells were washed twice with PBS and then treated with secondary antibodies for 1 hour at room temperature. The secondary antibodies used were Alexa fluor 488 donkey anti-rabbit or anti-mouse IgG (Invitrogen, 1:1,000) and Alexa fluor 555 donkey anti-rabbit or anti-mouse IgG (Invitrogen, 1:1,000). Nuclei were stained with 0.5 μg/ml DAPI (Sigma). Images were captured using a Nikon Eclipse TE2000-U/X-cite 120 EXFO microscope with a photometric CoolSnap HQ2 camera.

In Vitro Differentiation and Teratoma Assay

Generation of embryoid bodies and in vitro differentiation were performed as described elsewhere [Takahashi, K. et al., *Cell* 131, 861-72 (2007)]. For the teratoma assay, 3-5 million cells were injected under the kidney capsule of SCID mice. Thirty one days later the tumors were excised and fixed in 4% paraformaldehyde and histologically analyzed at the TSRI histology core facility. The use of SCID mice was approved by the UCSD animal research committee.

RT-PCR

Total RNA was extracted from cells using RNeasy minikit (Qiagen). cDNAs were synthesized according to product instructions using superscript III first strand synthesis kit (Invitrogen). Two microliters of the reaction product was used for 24-28 PCR cycles using respective primers. The sequences of the primers are described elsewhere [Takahashi, K. et al., *Cell* 131, 861-72 (2007)].

Flow Cytometry

For flow cytometry analysis, the cultures were mildly trypsinized and harvested from 6 well plates. The cells were washed and resuspended in FACS buffer (PBS, 2 mM EDTA, 2 mM HEPES, 1% FBS), and were analyzed on a FACS Calibur cytometer (Becton Dickinson, San Jose, Calif.) with the CellQuest program.

Example 6. Reprogramming of Human Fibroblast

We tested the TGFβ receptor inhibitor (SB431542), MEK inhibitor (PD0325901) and GSK-3 inhibitor (CHIR99021), on human fibroblast crl2097 that were retrovirally transduced with Oct4, Klf4, Sox2 and c-Myc, for their effects on reprogramming kinetics and efficiency. Conditions are the same as set forth in Example 5. Data were collected 8 days after compound treatment.

We tested the combinations of TGFβ receptor inhibitor (SB431542) and MEK inhibitor (PD0325901) for their effects on reprogramming kinetics and efficiency. We found that the combination of 2 μM SB431542 and 0.2-0.8 μM PD0325901 (e.g., 0.2 μM, 0.4 μM, or 0.8 μM) was most effective in enhancing reprogramming of human fibroblast crl2097 transduced with Oct4, Klf4, Sox2 and c-Myc. These conditions result in best colony morphology, and the resulting cells express pluripotent markers such as Oct4 and Nanog. Conditions with a lower or higher concentration of PD0325901 (e.g., 2 μM SB431542 and 0.1 μM PD0325901; 2 μM SB431542 and 1.6 μM PD0325901; 2 μM SB431542 and 3.2 μM PD0325901) result in fewer cells and/or smaller colonies.

We also tested the combinations of GSK-3 inhibitor (e.g., 2, 4, or 8 μM CHIR99021) and MEK inhibitor (0.2 μM PD0325901) for their effects on reprogramming kinetics and efficiency. We further tested the combination of TGFβ receptor inhibitor (2 μM SB431542), GSK-3 inhibitor (e.g., 2, 4, or 8 μM CHIR99021) and MEK inhibitor (0.2 μM PD0325901) for their effects on reprogramming kinetics and efficiency. These conditions result in a higher number of colonies as compared to the combinations of TGFβ receptor inhibitor (SB431542) and MEK inhibitor (PD0325901).

Example 7. Reprogramming of Human Primary Keratinocytes

The induced pluripotent stem cell (iPSC) technology, i.e. reprogramming somatic cells into pluripotent cells that closely resemble embryonic stem cells (ESCs) by introduction of defined factors, holds great potential in biomedical research and regenerative medicine (Takahashi, K., and

Yamanaka, S., *Cell* 126, 663-676 (2006); Takahashi et al., *Cell* 131, 861-872 (2007); Yu et al., *Science* 318, 1917-1920 (2007); Zhou et al., *Cell Stem Cell* 4, 381-384 (2009); Kim et al., *Cell Stem Cell* 4, 472-476 (2009); Maherali, N., and Hochedlinger, K., *Cell Stem Cell* 3, 595-605 (2009a); Daley et al., *Cell Stem Cell* 4, 200-201 (2009)). Various strategies have been developed to generate iPSCs with less or no exogenous genetic manipulations, which represent a major hurdle for iPSC's applications (Yamanaka et al., 2009; Saha, K., Jaenisch, R., *Cell Stem Cell* 5, 584-595 (2009)). Toward an ultimate goal of generating iPSCs with a defined small molecule cocktail that would offer significant advantages over genetic manipulations or more difficult-to-manufacture/use biologics, substantial efforts and progresses have been made in identifying chemical compounds that can functionally replace exogenous reprogramming transcription factors (TFs) and/or enhance reprogramming efficiency and kinetics (Shi et al., *Cell Stem Cell* 2, 525-528 (2008a); Shi et al., *Cell Stem Cell* 3, 568-574 (2008b); Huangfu et al., *Nat Biotechnol* 26, 795-797 (2008a); Huangfu et al., *Nat Biotechnol* 26, 1269-1275 (2008b); Silva et al., *Plos Bio* 6, e253. doi: 10.1371/journal. pbio. 0060253 (2008); Lyssiotis et al., *PNAS* 106, 8912-8917 (2009); Ichida et al., *Cell Stem Cell* 5, 491-503 (2009); Maherali, N., Hochedlinger, K., *Curr Biol* 19, 1718-1723 (2009b); Esteban et al., *Cell Stem Cell* 6, 71-79 (2010); Feng et al., *Cell Stem Cell* 4, 301-312 (2009)). However, further reducing the number of exogenous TFs has been extraordinarily challenging due to the facts that (1) most reprogramming enabling or enhancing conditions (e.g. exploiting specific cell type or using small molecules) are context dependent, i.e. such specific condition (e.g. a reprogramming small molecule) typically would be much less effective or even harmful in a different cell type with different exogenous factors and used in a different window of treatment; and (2) high throughput screening is technically challenging when the reprogramming efficiency and speed further decrease exponentially due to fewer exogenous TFs used. To date, only neural stem cells (NSCs) that endogenously express Sox2 and cMyc at high level were shown to be reprogrammed to iPSCs by exogenous expression of only Oct4 (Kim et al., *Cell* 136, 411-419 (2009a); Kim et al., *Nature* 461, 643-649 (2009b)). However, human fetal NSCs are rare and practically difficult to obtain (Nunes et al., *Nat Med* 9, 439-447 (2003)). Consequently, it would be essential to develop the reprogramming condition from other more accessible and abundant somatic cells. Here we report a novel small molecule cocktail that enables reprogramming of human keratinocytes to iPSCs with exogenous expression of only Oct4.

Among several readily available primary human somatic cell types, keratinocytes that can be easily isolated from human skin or hair follicle represent an attractive cell source for reprogramming, because they endogenously express Klf4 and cMyc, and were reported to be reprogrammed more efficiently using the conventional four TFs or three TFs (without Myc) (Aasen et al., *Nat Biotechnol* 26, 1276-1284 (2008); Maherali et al., *Cell Stem Cell* 3, 340-345 (2008)). More recently, we reported that dual inhibition of TGFβ and MAPK/ERK pathways using small molecules (i.e. SB431542 and PD0325901, respectively) provides a drastically enhanced condition for reprogramming of human fibroblasts with four exogenous TFs (i.e. OSKM) (Lin et al., *Nat Methods* 6, 805-808 (2009)). Furthermore, we have shown that such dual pathway inhibition could also enhance reprogramming of human keratinocytes by two exogenous TFs (i.e. OK) with two small molecules, Parnate (an inhibitor of lysine-specific demethylase 1) and CHIR99021 (a GSK3 inhibitor) (Li et al., *Stem Cells* 27, 2992-3000 (2009)). However, such 2-TFs reprogramming process was very inefficient and complex (e.g. involving two exogenous TFs and four chemicals), and reprogramming with even one less TF appeared daunting. Toward this end, we developed a step-wise strategy in refining reprogramming condition and identifying new reprogramming chemical entities. We first attempted to further optimize the reprogramming process under the four or three TFs (i.e. OSKM or OSK) condition in normal human epidermal keratinocytes (NHEKs) by testing various inhibitors of TGFβ and MAPK pathways at different concentrations using previously reported human iPSC characterization methods (Lin et al., *Nat Methods* 6, 805-808, (2009)). Encouragingly, we found that the combination of 0.5 μM PD0325901 and 0.5 μM A-83-01 (a more potent and selective TGFβ receptor inhibitor) was more effective in enhancing reprogramming of human keratinocytes transduced with OSKM or OSK. Remarkably, when we further reduced viral transductions to only two factors/OK, we could still generate iPSCs from NHEKs when they were treated with 0.5 μM PD0325901 and 0.5 μM A-83-01, although with low efficiency. We also found that 0.25 mM sodium butyrate (NaB, a histone deacetylase inhibitor) turned out to be much more reliable and efficient than the previously reported 0.5 mM VPA for the generation of iPSCs under OK condition.

Example 8. Reprogramming of Normal Human Epidermal Keratinocytes (NHEKs)

We tested the combination of HDAC inhibitor, GSK-3 inhibitor, TGFβ receptor inhibitor, MEK inhibitor on NHEKs that were lentivirally transduced with Oct4 alone, or Oct4 and Klf4, for their effects on reprogramming kinetics and efficiency.

Methods:

Normal Human Epidermal Keratinocytes (NHEKs, Lonza) were maintained in Keratinocyte culturing medium (KCM, Lonza). NHEKs were cultured in a 100 mm tissue culture dish and transduced 2-3 times (3 hours/time) with freshly produced lentivirus supernatants.

For NHEKs transduced with Oct4 and Klf4, 1,00,000 transduced NHEKs were seeded on the irradiated x-ray inactivated CF1 MEF feeder cells in a 100-mm dish and cultured in KCM and treated with GSK-3 inhibitor CHIR99021 (3 μM), HDAC inhibitor NaB (0.25 mM), and TGFβ receptor inhibitor A-83-01 (0.5 μM) for 2 weeks. Then cell culture media were changed to hESCM and supplemented with GSK-3 inhibitor CHIR99021 (3 μM), HDAC inhibitor NaB (0.25 mM), and TGFβ receptor inhibitor A-83-01 (0.5 μM) and MEK inhibitor PD0325901 (0.5 μM) for additional four weeks. The iPSC colonies were stained positive by Alexa Fluor 555 Mouse anti-Human TRA-1-81 antibody (BD Pharmingen). hESCM: DMEM/F12, 15% Knockout serum replacement, 1% Glutamax, 1% Non-essential amino acids, 1% penicillin/streptomycin, 0.1 mM β-mercaptoethanol and 10 ng/ml bFGF.

For NHEKs transduced with Oct4 alone, 1,000,000 transduced NHEKs were seeded on the irradiated x-ray inactivated CF1 MEF feeder cells in a 100-mm dish and cultured in KCM and treated with GSK-3 inhibitor CHIR99021 (3 μM), HDAC inhibitor NaB (0.25 mM), and TGFβ receptor inhibitor A-83-01 (0.5 μM) for 2 weeks, followed by changing half volume of media to hESCM and supplementing with GSK-3 inhibitor CHIR99021 (3 μM), HDAC inhibitor NaB (0.25 mM), and TGFβ receptor inhibitor A-83-01 (0.5 μM) for another 2 weeks. Then cell culture media were

changed to hESCM and supplemented with GSK-3 inhibitor CHIR99021 (3 μM), HDAC inhibitor NaB (0.25 mM), and TGFβ receptor inhibitor A-83-01 (0.5 μM) and MEK inhibitor PD0325901 (0.5 μM) for additional four weeks. The iPSC colonies were stained positive by Alexa Fluor 555 Mouse anti-Human TRA-1-81 antibody (BD Pharmingen). hESCM: DMEM/F12, 15% Knockout serum replacement, 1% Glutamax, 1% Non-essential amino acids, 1% penicillin/streptomycin, 0.1 mM β-mercaptoethanol and 10 ng/ml bFGF.

Results:

For NHEKs transduced with Oct4 and Klf4, we found that the treatment with the combination of 3 μM CHIR, 0.25 mM NaB, 0.5 μM A-83-01 and 0.5 μM PD0325901 results in a ~0.02% reprogramming efficiency. Reprogramming efficiency for Oct4 and Klf4 transduced NHEKs untreated with HDAC inhibitor, GSK-3 inhibitor, TGFβ receptor inhibitor, and MEK inhibitor is 0%.

For NHEKs transduced with Oct4 alone, we found that the treatment with the combination of 3 μM CHIR, 0.25 mM NaB, 0.5 μM A-83-01 and 0.5 μM PD0325901 results in a ~0.0004% reprogramming efficiency. Reprogramming efficiency for Oct4 transduced NHEKs untreated with HDAC inhibitor, GSK-3 inhibitor, TGFβ receptor inhibitor, and MEK inhibitor is 0%.

Example 9. Reprogramming of Human Umbilical Vein Endothelial Cells

We tested the combination of HDAC inhibitor, GSK-3 inhibitor, TGFβ receptor inhibitor, MEK inhibitor on HUVECs that were lentivirally transduced with Oct4 alone for their effects on reprogramming kinetics and efficiency.

Methods:

Human Umbilical Vein Endothelial Cells (HUVECs, Millipore) were maintained in EndoGRO-VEGF Complete Medium (HCM, CHEMICON). HUVECs were cultured in a 100 mm tissue culture dish and transduced 2 times (4-6 hours/time) with freshly produced lentivirus supernatants. Then 2,00,000 transduced HUVECs were seeded on gelatin coated 100-mm dish and cultured in HCM and treated with GSK-3 inhibitor CHIR99021 (3 μM), HDAC inhibitor NaB (0.25 mM), and TGFβ receptor inhibitor A-83-01 (0.5 μM) for 2 weeks, followed by changing half volume of media to hESCM and supplementing with GSK-3 inhibitor CHIR99021 (3 μM), HDAC inhibitor NaB (0.25 mM), and TGFβ receptor inhibitor A-83-01 (0.5 μM) for another 2 weeks. Then cell culture media were changed to hESCM and supplemented with GSK-3 inhibitor CHIR99021 (3 μM), HDAC inhibitor sodium butyrate (0.25 mM), and TGFβ receptor inhibitor A-83-01 (0.5 μM) and MEK inhibitor PD0325901 (0.5 μM) for additional 2 weeks. The iPSC colonies were stained positive by Alexa Fluor 555 Mouse anti-Human TRA-1-81 antibody (BD Pharmingen). hESCM: DMEM/F12, 15% Knockout serum replacement, 1% Glutamax, 1% Non-essential amino acids, 1% penicillin/streptomycin, 0.1 mM 3-mercaptoethanol and 10 ng/ml bFGF.

Results:

For HUVECs transduced with Oct4 alone, we found that the treatment with the combination of 3 μM CHIR, 0.25 mM NaB, 0.5 μM A-83-01 and 0.5 μM PD0325901 results in a 0.002% reprogramming efficiency. Reprogramming efficiency for Oct4 transduced HUVECs untreated with HDAC inhibitor, GSK-3 inhibitor, TGFβ receptor inhibitor, and MEK inhibitor is 0%.

Example 10. Reprogramming of Mouse and Human Somatic Cells without Sox2 Transgene

Here, we report that a specific GSK-3 inhibitor, CHIR99021, could allow the reprogramming of both mouse and human somatic cells without Sox2 transgene. Our studies suggest that the GSK-3 inhibitor might have a general application to replace transcription factors in both mouse and human somatic cell reprogramming.

Materials and Methods:

Cell Culture and Viral Transduction: MEFs were derived from 129S2/SvPasCrlf and ROSA26$^{+/-}$/OG2$^{+/-}$ mice according to the protocol reported on WiCell Research Institute website: "Introduction to human embryonic stem cell culture methods". ROSA26$^{+/-}$/OG2$^{+/-}$ heterozygous transgenic mice carry GFP reporter gene under the control of the Oct4 promoter (Oct4-GFP) and the ubiquitously expressed neo/lacZ transgene [Do J T, Scholer H R, Stem Cells, 22:941-949 (2004)]. Animal experiments were performed according to the Animal Protection Guidelines of the Max Planck Institute for Biomolecular Research, Germany. MEFs were transduced by Oct4, Klf4 and Sox2 three factors, or two-factor combinations of the pMXs-based retroviruses encoding mouse Oct4, Klf4 and Sox2 (Addgene) as previously described [Takahashi K, Yamanaka S, *Cell,* 126:663-676 (2006)]. Twenty four hours later, transduced MEFs were seeded in 6-well plate and incubated with mESC growth medium: Knockout™ DMEM, 7% ES Cell-Qualified fetal bovine serum, 10% Knockout Serum Replacement, 1% Glutamax, 1% Non-essential amino acids, 1% penicillin/streptomycin, 0.1 mM β-mercaptoethanol and 10^3 U/ml mLIF (Millipore). MEFs transduced with Oct4/Klf4/Sox2 (lx 10^4 cells/well together with 10^5 cells/well CF1 feeders in 6-well plates) were then treated with GSK-3 inhibitor CHIR99021 (Stemgent) for two weeks, and EGFP positive colonies were picked up at the third week after treatment. MEFs transduced with Oct4/Klf4 (lx 10^5 cells/well in 6-well plates) were treated with 10 μM CHIR99021 for four weeks, GFP positive colonies were picked up and expanded at the fourth to fifth week after treatment.

Normal Human Epidermal Keratinocytes (NHEKs, Lonza) were cultured and transduced with two-factor combinations of lentiviruses encoding human Oct4, Sox2 (p Sin-EF2-Puro-based) and mouse Klf4 (pLOVE-based) as previously described [Yu et al., *Science,* 318:1917-1920 (2007); Blelloch et al., Generation of Induced Pluripotent Stem Cells in the Absence of Drug Selection, 1:245-247 (2007)]. Lentiviral vectors were obtained from Addgene. Twenty four hours later, 1×10-transduced NHEKs were seeded on the irradiated X-ray inactivated CF1 MEF feeder cells in a 100 mm dish by keratinocyte medium (Lonza). One week after, the media was changed to human ES cell medium: DMEM/F12, 20% Knockout serum replacement, 1% Glutamax, 1% Non-essential amino acids, 1% penicillin/streptomycin, 0.1 mM β-mercaptoethanol and 100 ng/ml bFGF and treated with GSK-3 inhibitor CHIR99021 (Stemgent) (10 μM) alone or combined with valproic acid (0.5-2 mM), BIX-01294 (Stemgent) (1~2 μM), RG108 (Stemgent) (1-5 μM), Parnate (Sigma) (2-4 μM), PD0325901 (Stemgent) (0.5 μM) and SB431542 (Tocris) (2 μM). The media containing above small molecule combinations were changed every day. Two week after treatment, the cells were sub-cultured (1:1) on new feeder cells (PD0325901 and SB431542 were only used in the first two-week treatment). After another two weeks, the small molecules were removed and the cells were stained with Alexa Fluor 555-conjugated Mouse anti-Human TRA-1-81 antibody (BD Pharmingen).

The positive colonies were marked and picked up for expansion on feeder cells in human ES cell medium about 7 weeks after transduction. The human iPSCs were sub-cultured regularly by Accutase (Chemicon). All cell culture products were from Invitrogen/Gibco BRL except where mentioned.

Cytochemistry and Immunofluorescence Assay:

Alkaline Phosphatase staining was performed according to the manufacturer's protocol using the Alkaline Phosphatase Detection Kit (Millipore). For immunofluorescence assay, cells were fixed in 4% paraformaldehyde for 10 minutes and washed three times with PBS containing 0.1% Triton X-100 (Sigma-Aldrich). The fixed cells were then incubated in blocking buffer, 0.1% Triton X-100 and 10% normal donkey serum (Jackson ImmunoResearch Laboratories Inc) in PBS (Invitrogen/Gibco BRL), for 30 min at room temperature. The cells were then incubated with primary antibody overnight at 4° C. in blocking buffer. The day after, cells were washed with PBS and incubated with secondary antibody in PBS containing 0.1% Triton X-100 for one hour at room temperature. Mouse anti-Oct4 antibody (1:250) (Santa Cruz Biotechnology), rabbit anti-Sox2 antibody (1:2000) (Chemicon), mouse anti-SSEA1 antibody (1:250) (Santa Cruz Biotechnology), rabbit anti-Nanog antibody (1:250) (Abcam), rat anti-SSEA3 antibody (1:1000) (Chemicon), mouse anti-SSEA4 antibody (1:1000) (Chemicon), mouse anti-TRA-1-81 antibody (1:1000) (Chemicon), goat anti-Sox17 (1:200) (R&D), mouse anti-βIII-Tubulin (Tuj1) antibody (1:1000) (Covance Research Products), rabbit anti-Brachyury antibody (1:200) (Santa Cruz) were used as primary antibodies. Secondary antibodies were Alexa Fluor 486/555 donkey anti-mouse, anti-rat, anti-goat or anti-rabbit IgG (1:500) (Invitrogen). Nuclei were visualized by DAPI staining (Sigma-Aldrich). Images were captured using a Nikon Eclipse TE2000-U microscope.

Differentiation of iPSCs In Vitro:

The in vitro differentiation of miPSCs-OK and hiPSCs-OK was carried out by the standard embryoid body (EB) differentiation method. The iPSCs were dissociated by either 0.05% Trypsin-EDTA (miPSCs-OK) or Accutase (hiPSCs-OK), and then cultured in ultra-low attachment 100-mm dish in DMEM medium supplemented with 10% FBS to form EBs. The medium was changed every other day. One week later, the EBs were harvested and transferred into Matrigel-coated 6-well plate in DMEM medium with 10% FBS. Three to seven day later, the cells were fixed for immunocytochemistry analysis.

PCR Analysis:

To detect the expression of pluripotency genes by MEFs and NHEKs that were treated with small molecules, untransduced MEFs and NHEKs were treated for three days in mESC growth medium with 10 μM CHIR99021 or in hES cell medium with either combination of 10 μM CHIR99021 and 2 μM Parnate or combination of 10 μM CHIR99021, 2 μM Parnate, 0.5 μM PD0325901 and 2 μM SB431542. For the semi-quantitative and quantitative RT-PCR analyses, RNA was extracted from miPSCs-OK, hiPSCs-OK, MEFs, treated MEFs and treated NHEKs using the RNeasy Plus Mini Kit in combination with QIAshredder (Qiagene). Reverse transcription was performed with 1 μg RNA using iScript™ cDNA Synthesis Kit (BioRad). The expression of pluripotent markers by miPSCs-OK was analyzed by RT-PCR using Platinum PCR SuperMix (Invitrogen). The primers for the endogenous Oct4, Sox2, Klf4 and Nanog were as reported [Takahashi K, Yamanaka S, Cell, 126:663-676 (2006)]. Amplification of viral transduced genes was done using the gene specific forward primers (Klf4: 5'-GCG AAC TCA CAC AGG CGA GAA ACC-3' (SEQ ID NO:3); Sox2: 5'-GGT TAC CTC TTC CTC CCA CTC CAG-3' (SEQ ID NO:4) and Oct4: 5'-TTG GGC TAG AGA AGG ATG TGG TTC-3' (SEQ ID NO:5)) and common reverse primer pMXs-L3205 (5'-CCC TTT TTC TGG AGA CTA AAT AAA-3' (SEQ ID NO:6)) [Takahashi et al, *Cell,* 131:861-872 (2007)]. The RT-PCR was performed in 30 (amplification of pluripotent markers) or 35 (amplification of viral transduced genes) cycles (94° C. for 30 s, annealing temperature for 30 s, and 72° C. for 30 s). Real-time PCR was carried out using iQ SYBR Green Supermix (BioRad). The primers for the human endogenous Oct4, total Oct4, endogenous Sox2, total Sox2, Nanog, Klf4, GDF-3 and Cripto were as reported [Yu et al., *Science,* 318:1917-1920 (2007); Aasen et al., *Nat Biotechnol.,* 26:1276-1284 (2008); Mateizel et al., *Hum Reprod.,* 21:503-511 (2006) 4, 26, 27]. The primer for viral Klf4 was 5'-CAC CTT GCC TTA CAC ATG AAG AGG-3' (SEQ ID NO:7) and 5'-CGT AGA ATC GAG ACC GAG GAG A-3' (SEQ ID NO:8). The primer for FGF-4 was 5'-GAC ACC CGC GAC AGC CT-3' (SEQ ID NO:9) and 5'-TCA CCA CGC CCC GCT-3' (SEQ ID NO:10). The expression of genes of interest was normalized to that of GAPDH in all samples.

Genomic DNA was extracted from miPSCs-OK using DNeasy Blood & Tissue Kit (QIAGEN). In order to analyze the viral integration in miPSCs-OK, the genomic DNA of miPSCs-OK was subjected to PCR analysis using the same primers employed to amplify the viral transduced genes in the RT-PCR experiments. For the methylation analysis of Oct4 promoter by bisulfite-sequencing, DNA samples from hiPSC-OK were isolated using the Non Organic DNA Isolation Kit (Millipore) and were then treated with the EZ DNA Methylation-Gold Kit (Zymo Research Corp., Orange, Calif.). The treated DNA samples were then used as templates to amplify targets of interest. Primers used for the amplification of the Oct4 promoter fragment (406 bp, from −2192~−1786) were 5'-GGA TGT TAT TAA GAT GAA GAT AGT TGG-3' (SEQ ID NO:11) and 5'-CCT AAA CTC CCC TTC AAA ATC TAT T-3' (SEQ ID NO:12) [Deb-Rinker et al., *J. Biol. Chem.,* 280:6257-6260 (2005)]. The resulting fragments were cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen) and sequenced.

Aggregation of iPSCs with Zona-Free Embryos:

miPSCs-OK were aggregated with denuded post-compacted eight-cell stage embryos to obtain aggregate chimeras. Eight-cell embryos (B6C3F1) were flushed from females at 2.5 dpc and cultured in microdrops of KSOM medium (10% FCS) under mineral oil. Clumps of iPSCs (10-20 cells) after short treatment of trypsin were chosen and transferred into microdrops containing zona-free eight-cell embryos. Eight-cell embryos aggregated with iPSCs were cultured overnight at 37° C., 5% CO_2. Aggregated blastocysts that developed from eight-cell stage were transferred into one uterine horn of a 2.5 dpc pseudopregnant recipient. The recipient mice were sacrificed at ED 13.5 day. The embryos were analyzed by x-gal staining to reveal the contribution of iPS cells.

Teratoma Formation:

Three to five million hiPSC-OK (passage 8, clone 1) were injected under the kidney capsule of SCID mice (n=3). After 6-8 weeks, the neoplasm was removed and then histologically analyzed.

Results

CHIR99021 can Significantly Promote the Reprogramming of MEFs Transduced by Oct4, Sox2 and Klf4.

It had been shown that Oct4/Sox2/Klf4-infected MEFs could be reprogrammed into pluripotent state with higher

efficiency when cultured under Wnt3a-conditioned medium [Marson et al., *Cell Stem Cell,* 3:132-135 (2008)]. However, small molecule activators of Wnt signaling pathway were not found to have similar effects. Combination of CHIR99021, a GSK-3 inhibitor which can activate Wnt signaling pathway, with PD0325901, a MEK inhibitor, was shown to promote partially reprogrammed iPSCs to full pluripotency [Silva et al., *PLoS Biol.,* 6:e253 (2008)]. Concurrent with those studies, we found that CHIR99021 could significantly promote reprogramming of murine fibroblasts. Treating Oct4/Sox2/Klf4 transduced MEFs with CHIR99021 for two weeks significantly increased the number of alkaline phosphase (AP)-positive mESC-like colonies in a dose-dependent manner (AP staining was done at the third week after treatment) (FIG. **8**A). CHIR99021 treatment of Oct4/Sox2/Klf4-transduced MEFs (ROSA26$^{+/-}$/OG2$^{+/-}$), which express GFP under the control of Oct4 promoter and also ubiquitously LacZ, also increased the number of GFP-positive colonies, which could be observed as early as two weeks after treatment. CHIR99021 showed the greatest effects at about 10 μM, which increase efficiency from 0.03-0.08% to 0.2-0.4% of transduced MEFs. (FIG. **8**B). Our results therefore suggest that CHIR99021 can significantly improve reprogramming efficiency of MEFs transduced with Oct4, Sox2, and Klf4. These mouse iPS cell colonies could be stably expanded under conventional mESC growth condition and express typical pluripotency markers, such as AP, Oct4, Sox2, Nanog, SSEA1 by cytochemistry and immunostaining.

CHIR99021 enabled the reprogramming of MEFs transduced by Oct4/Klf4. We had previously identified BIX01294, a small molecule inhibitor of a histone methyltransferase G9a, which enabled reprogramming of both mouse NPCs and MEFs infected by only Oct4 and Klf4 [Shi et al., *Cell Stem Cell.,* 2:525-528 (2008); Shi et al., *Cell Stem Cell,* 3:568-574 (2008)]. We then investigated whether iPS cells could be generated from MEFs with fewer reprogramming factors in the presence of CHIR99021. OG2 MEFs transduced with different two-factor combinations (Oct4/Klf4, Oct4/Sox2 and Sox2/Klf4) were treated with 10 μM CHIR99021. GFP positive iPS cell colonies were identified only when MEFs were transduced with the combination of Oct4 and Klf4, but not with any other combination. On average, about six GFP positive colonies were identified out of 10^5 0 G2 MEFs 4~5 weeks after Oct4/Klf4 transduction and CHIR99021 treatment. Stable iPS cell lines (miPSC-OK) were established by picking up the GFP positive colonies (FIG. **9**A, **9**B). Immunocytochemistry revealed that miPSC-OK express typical pluripotency markers, such as Oct4, Sox2, Nanog and SSEA-1 (FIG. **9**C-F). MEFs do not express Sox2 endogenously, and real-time PCR analysis revealed that CHIR99021 treatment did not induce the expression of Sox2 and Oct4 in MEFs (FIG. **9**G). Therefore, the mechanisms by which CHIR99021 promotes the reprogramming of MEFs transduced by Oct4/Klf4 are independent of direct Sox2 induction.

RT-PCR analysis confirmed the reactivation and expression of the endogenous mouse Oct4, Sox2, Nanog, and Klf4 (FIG. **10**A). By using the specific primers for transgenes, RT-PCR analysis revealed that the viral genes were largely silenced (FIG. **10**A). PCR of genomic DNA of miPSC-OK confirmed the integration of retroviral Oct4 and Klf4, but no other reprogramming genes (FIG. **10**B). To examine the developmental potential of miPSC-OK, in vitro differentiation assay was preformed. Immunostaining showed miPSC-OK could differentiate into endoderm (Sox17), mesoderm (brachyury) and neuroectoderm (βIII-tubulin) derivatives under the standard embryoid body differentiation methods (FIG. **10**C-E). Most importantly, miPSC-OK (clone1 and clone2) could efficiently incorporate into the inner cell mass (ICM) of blastocysts after aggregation with 8-cell embryos, led to mid-gestational chimerism (ED 13.5) after the aggregated embryos were transplanted into mice, and contributed to germline cells in vivo (FIG. **10**F-H). Twenty transplanted embryos aggregated with miPSC-OK (clone 1) were allowed to be born. However, no chimeric mice are identified from the living pups. These in vitro and in vivo characterizations confirm that the miPSC-OK are molecularly, morphologically, and functionally similar to the original four-factor iPS cells and the mouse ESCs.

CHIR99021 enabled the reprogramming of normal human keratinocytes transduced with Oct4/Klf4 when combined with Parnate. We next investigated whether human iPS cells could be generated with fewer transcription factors in the presence of CHIR99021 and/or direct epigenetic modifiers including inhibitors of DNA methyltransferase (RG108), histone methyltransferase (BIX01294), histone deacetylase (valproic acid/VPA) and lysine-specific demethylase 1 (Parnate). To this end, we selected primary normal human epidermal keratinocytes (NHEKs), concurrent with recent studies suggesting that keratinocytes transduced with four factors could be reprogrammed into iPS cells more efficiently and rapidly in comparison to other somatic cell types [Aasen et al., *Nat Biotechnol.,* 26:1276-1284 (2008)]. Primary keratinocytes were transduced with different two-factor combinations (Oct4/Klf4, Oct4/Sox2 and Sox2/Klf4), treated with CHIR99021 alone, or combined with epigenetic modifiers, and then stained with the human pluripotency cell-surface marker TRA-1-81 5 weeks post-infection. Tra-1-81 positive human ESC-like colonies could only be identified from culture infected by Oct4 and Klf4 in the presence of CHIR99021 and Parnate. On average, about two Tra-1-81 positive colonies could be identified out of 10^5 transduced HNEKs, which was at least 100 times less efficient than 4-factor transduced keratinocytes. Stable human iPS cells could be established and long-term expanded by picking up these colonies (named hiPSC-OK). In addition, we have also found that combined treatment using inhibitors of MEK (PD0325901) and TGFβ receptor (SB431542) could improve the reprogramming efficiency of human fibroblasts transduced by Oct4/Sox2/Klf4/c-Myc (unpublished data). By using CHIR99021 (10 μM) and Parnate (2 μM) as the basal condition, addition of PD0325901 (0.5 μM) and SB431542 (2 μM) could further increase the TRA-1-81 positive colonies from human keratinocytes transduced with Oct4/Klf4 (about 5-10 Tra-1-81 positive colonies could be identified out of 10^5 transduced HNEKs), but the detailed mechanisms underlying this observation still need to be revealed. Nine TRA-1-81 positive colonies were expanded, and four stable human iPS cells (hiPSC-OK), one from CHIR99021 and Parnate condition (hiPSC-OK 1) and another three from CHIR99021/Parnate plus PD0325901/SB431542 condition (hiPSC-OK 2-4), were further studied and long-term cultured for over 20 passages. hiPSC-OK express typical pluripotency markers, such as AP, Oct4, Sox2, Nanog, TRA-1-81, SSEA3 and SSEA-4 (FIG. **11**A-D). Real-time PCR analysis confirmed expression of the endogenous human Oct4, Sox2, Nanog, Cripto, GDF-3 and FGF4 (FIG. **11**E). Although the viral Oct4 and Klf4 expression was not completely silenced, bisulfite sequencing analysis revealed that the Oct4 promoter of hiPSC-OK is largely demethylated (FIG. **11**F). Similar to the CHIR99021 treatment of MEFs, real-time PCR analysis indicated neither CHIR99021/Parnate (2 inhibitors) nor CHIR99021/Parnate/

PD0325901/SB431542 (4 inhibitors) treatment induced the expression of Sox2 and Oct4 in keratinocytes immediately (FIG. **12**). The terminal differentiation of keratinocytes induced by the human ES cell culture media may result in the significant down-regulation of c-Myc expression after treatment (FIG. **12**).

To examine developmental potentials of hiPSC-OK, in vitro differentiation assays were preformed. Immunostaining confirmed that hiPSC-OK could differentiate into endoderm (Sox17), mesoderm (brachyury) and neuroectoderm (βIII-tubulin) (FIG. **13**A-C) derivatives in vitro. Furthermore, after transplanted into the SCID mice, hiPSC-OK formed teratoma consisting of representative derivatives of all three germ layers including epithelial tube structure (endoderm) (FIG. **13**D), cartilage-like structure (mesoderm) (FIG. **13**E) and neuroepithelium-like structure (ectoderm) (FIG. **13**F). These in vitro and in vivo characterizations confirm that the human iPS cells generated by Oct4 and Klf4 viral transduction closely resemble human ES cells in terms of typical pluripotency marker expression and differentiation potential.

Discussion

Reprogramming is a very slow and inefficient process. Such low efficiency and slow kinetics also present "hidden" risks in iPSCs, such as accumulated and selected subtle genetic and epigenetic abnormalities. Consequently, it is highly desirable to identify new conditions/small molecules that can promote reprogramming and/or replace certain factors.

In the present study we reported that the GSK-3 inhibitor CHIR99021 can significantly improve the reprogramming efficiency of MEFs transduced by Oct4/Sox2/Klf4, and also enable the reprogramming of MEFs transduced by only Oct4 and Klf4. When combined with Parnate, CHIR99021 can result in the reprogramming of human primary keratinocytes transduced with only Oct4 and Klf4 as well. Although previous studies showed that the activation of Wnt signaling promote somatic cell reprogramming, this study is the first report to show GSK-3 inhibitor could allow the reprogramming of both mouse and human somatic cell without Sox2. Recently it is reported that the target genes co-bounded by Oct4, Sox2 and Klf4 in ES cells showed a lower histone H3 lysine 4 (H3K4) trimethylation enrichment in partially reprogrammed cells than in ES/iPS cells, and this low histone H3K4 trimethylation may result in the lack of binding of many important regulators of pluripotency by Oct4, Sox2 and Klf4 [Sridharan et al., *Cell,* 136:364-377 (2009)]. Parnate, a monoamine oxidase inhibitor used as an antidepressant drug, showed potent inhibitory effect on lysine-specific demethylase 1 and inhibiting the H3K4 demethylation, but does not influence the acetylation of H3K9/K14 [Mimasu et al., *Biochem Biophys Res Commun.,* 366: 15-22 (2008)]. Parnate may facilitate the full reprogramming of HNEK transduced with only Oct4 and Klf4 by inhibiting H3K4 demethylation.

Particularly, this is also the first time to generate human iPS cells from somatic cells without exogenous Sox2 expression. Both Oct4 and Sox2 are critical regulators in human/mouse ES cell pluripotency and also the only common reprogramming factors used for generation of human iPS cells. Replacement of Sox2 in human cell reprogramming represents an important step toward identifying a chemically defined condition that could allow reprogramming human somatic cells by Oct4 only or without the forced expression of any exogenous factor. As HNEKs express Klf4 endogenously, it would be conceivable that HNEKs could perhaps be fully reprogrammed with only Oct4 transduction, but so far it was not achieved for unknown reasons. When Oct4 transduced HNEKs were treated under the same chemical condition, some human ES cell-like colonies were observed. After picking up these colonies, stable lines were established which could be long-term cultured under conventional human ES cell media. However, these cells are negative to AP staining, and expression of other pluripotency markers, such as Nanog and Sox2 could not be detected by immunostaining.

Our studies underscore the unique advantage of the chemical approach for improving reprogramming that may ultimately allow the generation of iPS cells or multipotent tissue-specific cells in completely chemically defined conditions without any permanent genetic modification. Ultimately, a completely chemically defined condition for efficient reprogramming of somatic cells would be highly favorable for various iPS cell applications.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, databases, Genbank sequences, patents, and patent applications cited herein are hereby incorporated by reference.

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What is claimed is:

1. A composition comprising:

(i) a mammalian non-pluripotent cell comprising exogenous polynucleotides comprising an Oct4 encoding polynucleotide and a Klf encoding polynucleotide; and

(ii) a small molecule composition comprising a GSK-3 inhibitor.

2. The composition of claim **1**, wherein the small molecule composition further comprises a MEK inhibitor, an Erk inhibitor, or a TGFβ receptor/ALK5 inhibitor.

3. The composition of claim **2**, wherein the TGFβ receptor/ALK5 inhibitor is selected from the group consisting of: A-83-01 and SB431542.

4. The composition of claim **2**, wherein the MEK inhibitor is PD0325901.

5. The composition of claim **1**, wherein the GSK-3 inhibitor is selected from the group consisting of: CHIR99021, CHIR98014, and 6-bromoindirubin-3'-oxime (BIO).

6. The composition of **1**, wherein the mammalian non-pluripotent cell is contacted with exogenous polynucleotides comprising an Oct4 encoding polynucleotide and a Klf4 encoding polynucleotide to initiate reprogramming.

7. A composition comprising: an induced pluripotent stem cell reprogrammed from a mammalian non-pluripotent cell, and a small molecule composition comprising a GSK-3 inhibitor.

8. The composition of claim **7**, wherein the small molecule composition further comprises a MEK inhibitor, an Erk inhibitor, or a TGFβ receptor/ALK5 inhibitor.

9. The composition of claim **8**, wherein the TGFβ receptor/ALK5 inhibitor is selected from the group consisting of: A-83-01 and SB431542.

10. The composition of claim **8**, wherein the MEK inhibitor is PD0325901.

11. The composition of claim **7**, wherein the GSK-3 inhibitor is selected from the group consisting of: CHIR99021, CHIR98014, and 6-bromoindirubin-3'-oxime (BIO).

12. The composition of claim **8**, wherein the induced pluripotent stem cell is reprogrammed by introducing into mammalian non-pluripotent cell exogenous polynucleotides comprising an Oct4 encoding polynucleotide and a Klf encoding polynucleotide to initiate reprogramming.

* * * * *